Dissecting the mechanisms of Regulatory T cell-derived Extracellular Vesicle (EV)-mediated suppression to facilitate the optimisation of these cells and EVs in the clinic

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Dissecting the mechanisms of Regulatory T cell-derived Extracellular Vesicle (EV)-mediated suppression to facilitate the optimisation of these cells and EVs in the clinic

A thesis submitted to King's College London for the degree of

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by

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Declaration

The work described in this thesis is my own original research that I carried out personally, unless otherwise stated. All sources of information are acknowledged by means of reference.

Sim Lai Tung

Abstract

Regulatory T cells (Tregs) are currently being clinically assessed as a means to treat various immune conditions including transplant rejection. Despite their clinical application, Treg suppression mechanisms remain to be fully elucidated. Recently, the host laboratory discovered a novel mechanism of suppression by mouse Tregs; the release of immunomodulatory extracellular vesicles (EVs). These vesicles are released by many cell types and their contents specifically packaged, with bioactive material, to mediate cell-to-cell communication. Transfer of their contents into target cells modulates their functions.

The goals of my research was to test whether; 1) mouse Treg EVs modulate bone-marrow derived dendritic cells (BM-DCs) function through the transfer of microRNAs (miRNAs); 2) human Tregs release EVs and investigate their miRNA contents; 3) human Treg EVs modulate T responder cell (Tresps) functions *in vitro*; 4) human Treg EVs can function *in vivo* to protect against transplant rejection.

My results have shown that mouse Treg EVs specifically contained miR-142-3p and miR-150-5p; and when co-cultured with BM-DCs, the expression levels of these miRNAs were increased in BM-DCs. miR-142-3p and miR-150-5p have been reported to modulate DC cytokine profiles suggesting that Treg EVs can affect the aforementioned. Indeed, mouse Treg EVs reduced IL-6 whilst increased IL-10 production levels by BM-DCs, perhaps to promote an anti-inflammatory milieu. Human Tregs, upon activation released ~120nm sized cup-shaped EVs. These human Treg EVs suppressed Tresps proliferation and modulated their cytokine production profiles by inhibiting pro-inflammatory IFNγ, IL-2 and IL-6 cytokine production levels whilst promoting the release of IL-10 and IL-4 by Tresps. Exclusive miRNAs which were absent in human Tregs but enriched in human Treg EVs included; miR-369-3p, miR-376c-3p and miR-195-3p, which were predicted to target 3'-untranslated regions (3'-UTR) of IFNγ, IL-2 and IL-6 mRNA. Importantly, human Treg EVs functioned *in vivo* to protect against human skin transplant damage by reducing immune cell infiltration of alloreactive $CD3^+$ T cells, $CD45^+$ and $Ki67^+$ cells within the transplanted allograft. The results generated herein demonstrated that human Treg EVs are functionally immunosuppressive and can protect against transplant rejection, suggesting that Treg EVs may in the future be a clinically applicable cell-free therapy for transplant patients.

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Dedicated to my beloved family

For their love, care, support and sacrifices

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List of Abbreviations

4-OHT= 4-hydrotamoxifen

41BB= tumour necrosis factor receptor superfamily member 9

ACK= ammonium chloride potassium

AICD= Activation-induced cell death

ADP= adenosine diphosphate

AMP= adenosine monophosphate

AP-1= Activator Protein-1

APC= Antigen Presenting Cell

ATP= adenosine triphosphate

ATRA= all-trans retinoic acid

B6= C57BL/6

Bcl-2= B-cell lymphoma 2

Bcl-xL= B-cell lymphoma-extra large

BCR= B Cell Receptor

Bim= Bcl-2-like protein 11

BM-DCs**=** bone marrow-derived dendritic cells

BRG**=** BALB/c $RAG2^{-/-}$ $\gamma c^{-/-}$

BSA= bovine serum albumin

c-SMAC= central supramolecular activation complex

cAMP= cyclic adenosine monophosphate

CBA= cytokine bead array

CCL= chemokine ligand

CCR= chemokine receptor

CD= cluster of differentiation

CD40L= CD40 Ligand

CLA= cutaneous lymphocyte-associated antigen

CLP= common lymphoid progenitor

CNI= Calcineurin Inhibitor

Cpm= counts per minute

CTL= cytotoxic T lymphocyte

CTLA-4= Cytotoxic T lymphocyte associated protein 4

CTV= CellTrace Violet

CXCR4= C-X-C chemokine receptor type 4

DC= Dendritic Cell

Dicer KO= Rosa26-ERT2-Cre Dicer$^{floxed/floxed}$

DMEM= Dulbecco's Modified Eagle medium

DN= double negative

DNA= deoxyribonucleic acid

DP= double positive

DTH= delayed-type hypersensitivity

EAE= experimental autoimmune encephalomyelitis

EBV**=** Epstein-Barr virus

EQ**=** ExoQuick-TC™

ESCRT= endosomal sorting complexes required for transport

EVs= Extracellular Vesicles

FACS= fluorescence-activated cell sorting

FasL= Fas Ligand

FCS= fetal calf serum

FDR= False discovery rate

FKBP12= FK506-binding protein 12

FoxP3= forkhead box P3

GITR= glucocorticoid-induced TNFR-related protein

GM-CSF= granulocyte macrophage colony-stimulating factor

GMP= good manufacturing practice

GvHD= graft versus host disease

Gy= Gray

HEK293Ts= Human embryonic kidney 293T

HLA= Human Leukocyte Antigen

HSP= Heat shock protein

IBD= inflammatory bowel disease

ICAM-1= intercellular adhesion molecule 1

ICOS= inducible T cell costimulatory

IFNγ= Interferon gamma

IKK2= inhibitor of nuclear factor kappa-B kinase subunit beta

IL= interleukin

IL-2R= Interleukin-2 Receptor

iNOS= nitric oxide synthase

IPEX= immunodysregulation polyendocrinopathy enteropathy X-linked

iTregs= *in vitro*-induced regulatory T cell

IU= international units

Lag-3= lymphocyte-activation gene 3

LB= Lysogeny broth

LFA-1= leukocyte function-associated antigen-1

LPS= lipopolysaccharide

MHC= Major Histocompatibility Complex

miRNA= micro-RNA

MMF= mycophenolate mofetil

MMP1a= matrix metallopeptidase 1a

mRNA= messenger ribonucleic acid

MS= multiple sclerosis

MSCs= mesenchymal stem cells

mTOR= Mammalian Target of Rapamycin

MVB= multivesicular bodies

MVE= multivesicular endosome

NF-kB= nuclear factor kappa-light-chain-enhancer of activated B cells

NK= natural killer

ns= non-significant

nSMase= neutral sphingomyelin phosphodiesterase

NTA= nanoparticle tracking analysis

OCT= optimal cutting temperature

OVA= ovalbumin

OX40= tumour necrosis factor receptor superfamily member 4

PBS= phosphate buffered saline

PD-1= programmed cell death protein 1

PEI= Polyethylenimine

PMID= PudMed Indentification

PPL= project licence

pTregs= peripheral-derived regulatory T cell

Rab27DKO= Rab27$\alpha^{ashen/ashen}$ $\beta^{-/-}$ knock-out

RAG-2= recombinant-activating gene 2

RBCs= Red blood cells

RNA= ribonucleic acid

RNase= ribonuclease

RPMI= Roswell Park Memorial Institute

SD= standard deviation

SEM= standard error of the mean

SLE= systemic erythematosus

SOC= super optimal broth with catabolite repression

SP= single positive

TAE= Tris-acetate-EDTA

Tconv= Conventional T cell

TCR= T Cell Receptor

Teff= T effector cell

TEMS= tetraspanin enriched microdomains

TGF-β= Transforming growth factor beta 1

Th= T helper

TIM-3= T cell immunoglobulin and mucin-domian containing-3

TNF= tumour necrosis factor

TNFR= tumour necrosis factor receptor

Tr1= Type-1 regulatory T cell

TRAIL= TNF-related apoptosis-inducing ligand

Treg= Regulatory T cell

Tresps= T responder cells

tRNA= transfer RNA

TSDR= Treg-specific demethylated region

Tsg101= tumour susceptibility gene 101

tTregs= thymus-derived regulatory T cell

VDJ= variable diversity joining

Chapter 1
General Introduction

Chapter 1 - General Introduction

1.1 Transplantation

Transplantation is the medical and surgical process of removing organs or tissues from the donor body to be placed and retained inside a recipient body with the purpose of replacing damaged, missing or suboptimal organs and tissues of the recipient. Although transplantation is the gold standard treatment for patients with end-stage organ failure by providing them with a better quality of life, it is still hindered by transplant rejection (Pervinder Sagoo, Lombardi, and Lechler 2012; Safinia et al. 2013).

Transplant or allograft rejection is a complex process that occurs when the recipient's immune cells identifies the donor antigen as 'foreign' similar to them identifying bacteria or viruses as harmful and thus initiates an immune response to destroy the donor allograft, ultimately leading to allograft dysfunction and failure (Pervinder Sagoo, Lombardi, and Lechler 2012).

Existing protocols to manage allograft rejection include a life-long immunosuppressive drug regimen which can mitigate acute rejection (Ali et al. 2013), but due to their non-specific immune suppression the patient can suffer from severe side effects. Nephrotoxicity, neurotoxicity, increased risk of developing cancer and increased susceptibility to acquiring infections are examples of these severe side effects (Salvadori and Bertoni n.d.; D. Hsu and Katelaris 2009). Notwithstanding, patients have survival rates of above 90% one year post-transplantation, however, the long-term acceptance of the allograft is still challenging due to chronic rejection (Romano et al. 2017). Since immunosuppressive drugs have minimal effectiveness to impede chronic rejection, the half-life of allografts remain limited to approximately 10-15 years (Gruessner and Gruessner 2013; Burgos et al. 2012). Another caveat associated with transplantation is the inadequate supply of donor organs to meet demands (Testa and Siegler 2014) and thus many patients unfortunately die before receiving a potentially life-saving organ.

Despite these multiple impediments, several treatments are under investigation as a prospective means of inducing a state of 'transplant tolerance', whereby transplanted tissues are accepted indefinitely. Ultimately, this could negate the need for life-long

immunosuppression or the heavy dependency of adequate, available and suitable donor organs.

1.2 Brief History of Transplantation

British biologist and Nobel laureate Sir Peter Medawar (whom received a shared Nobel Prize in 1960 for his work on acquired immunological tolerance (Starzl 1995)) researched extensively on graft rejection, initially in human studies together with Gibson in 1943 (Gibson and Medawar 1943) and later in 1944 with rabbit studies (Medawar 1944), which lead to the discovery that rejection of the skin was an immunological phenomenon. By 1953, Billingham, Brent and Medawar published an article in Nature describing the novel discovery of acquired immune tolerance which is an active suppression of the immune system activity towards antigens (Billingham, Brent, and Medawar 1953). Since Sir Peter Medawar's discoveries, it opened the experimental arena for many researchers searching for various strategies to dampen the recipient immune system (Starzl 1995). Table 1.1 summaries the first successful major transplant surgeries performed in human.

Year	Surgeons	Human Organ	Donor/Recipeint details	Notes	Reference
1954	Joseph Murray	Kidney	Recipient patient's own identical twin	Bypassed complication of transplant rejection as identical twin	PMID:11317972 PMID:13278189
1963	James Hardy	Lung	Recipient patient's arterial oxyegn saturation was enchanced from 87% to 98%	Patient received prednisone, azathioprine and colbalt radiation to the mediastinum and thymus	PMID:14061414
1967	Thomas Starzl	Liver	Liver was functioning in recipient patients	Patients received antilymphocytic serum treatment	PMID: 4877589
1967	Christian Narnard	Heart	Transplanted heart was functionally contracting in the recipient	Patient died of pneumonia 18 days later	PMID:4170370

Table 1.1- The first major achievements in human transplantation

The outcome of transplantation has undeniably improved in recent times compared to historical surgeries, most attributable to enhanced surgical techniques including improved vascular anastomoses skills, sterility procedures, better preservation chemicals to improve prolonged periods of *ex vivo* donor organ storage whilst still preserving their functions and enhanced post-surgery medical treatments including modern immunosuppressive regimens (Enderby and Keller 2015; Kellar 2015). However, three key problems linked to transplantation remain which are the limited availability of

suitable organs, the long-term use of immunosuppressive drugs causing toxicity and a large percentage of transplants are chronically rejected despite immunosuppression.

1.3 Pathways of Allorecognition

It is vital that immune cells can distinguish between 'self' and 'non-self' cells to ensure that invading foreign pathogens are destroyed whilst tolerance to self-antigens are continued to maintain normal immune function. In the context of transplantation, the presence of 'non-self' cells inside a recipient can elicit an immune response, known as 'immunogenicity'. This is initiated when the immunological identification of histo-incompatible antigens derived from the same species although from genetically disparate individuals occurs via the molecular engagement of a receptor-ligand system (T cell receptor (TCR)/ B cell receptor (BCR) - major histocompatibility complex (MHC)), a process termed 'allorecognition'. The ensuing immune cell activation and responses lead to allograft rejection, a process known as the 'alloresponse' (Afzali, Lombardi, and Lechler 2008; Game and Lechler 2002; Benichou and Thomson 2009).

The surgical procedure of organ transplantation is invasive to the patient and the donor organ is susceptible to ischemia/reperfusion injury, inflammatory damage and tissue stress. As a result, innate immune responses such as the complement system initiate the early process of allograft rejection. Nonetheless, studies performed on neonatally thymectomised (Miller 1961) and irradiated adult murine models (Hall, de Saxe, and Dorsch 1983; Hall, Dorsch, and Roser 1978a, 1978b) have revealed that recipient T cells are one of the most deleterious cell types that drive allograft rejection, therefore understanding these mechanisms could provide novel targets for intervention in transplantation therapy.

These recipient T cells can recognise alloantigens via three known and distinct but not mutually exclusive pathways of allorecognition; the direct, indirect and semi-direct pathways (Figure 1.1). The direct pathway of allorecognition is one that donor antigen presenting cells (APCs) present intact allogeneic MHC-allopeptide complexes to recipient T cells. On the other hand, the indirect pathway of allorecognition occurs when recipient APCs encounter, process and present allopeptides in the context of recipient-MHC class II molecules. The semi direct pathway of allorecognition combines

features of both 'direct' and 'indirect' pathways. This pathway is when the recipient APCs acquires and presents allogeneic MHC-allopeptide complexes to recipient T cells.

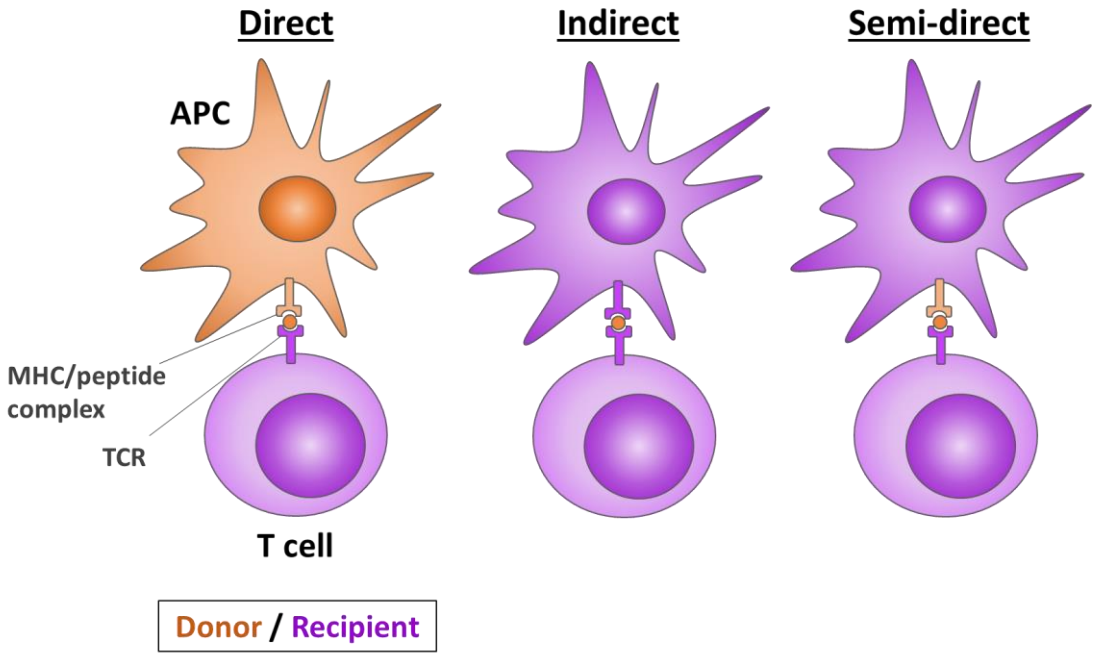


Figure 1.1- Pathways of allorecognition

Schematic diagram illustrating the three distinct pathways of allorecognition. The direct pathway of allorecognition is one that donor antigen presenting cells (APCs) present intact allogeneic MHC-allopeptide complexes to recipient T cells. On the other hand, the indirect pathway of allorecognition occurs when recipient APCs encounter, process and present allopeptides in the context of recipient-MHC class II molecules. The semi direct pathway of allorecognition combines features of both 'direct' and 'indirect' pathways. This pathway is when the recipient APCs acquires and presents allogeneic MHC-allopeptide complexes to recipient T cells.

1.3.1 Direct

'Passenger' leukocytes which reside in the allograft are a major source of tissue immunogenicity (Snell 1957). In particular, the high presence of donor APCs inherent in the allograft initiates rejection via the direct pathway (G. Yu et al. 2006). Additionally, this pathway pre-dominates the acute phase of the alloresponse. The robustness of the

direct allorecognition is highlighted by the exceptionally high frequency of T cell reactivity against allopeptides presented by allogeneic-MHC molecules (approximately 1-10%) (Lindahl and Wilson 1977; Whitelegg et al. 2005) compared to T cells which recognise allopeptides presented in the context of self-MHC molecules (approximately 0.01%) (Veerapathran et al. 2011; Ford and Burger 1983). Key studies which indicated that dendritic cells (DCs) were the principal APCs mediating priming of naïve T cells against allograft antigens was published in 1982 by Lechler and Batchelor (Lechler and Batchelor 1982b, 1982a). They performed experiments on rat kidneys whereby the kidneys were temporarily 'parked' in intermediate recipients to eliminate passenger leukocytes before being transferred into another recipient. The result was an enhanced allograft survival rate which was reversed by the repletion of donor DCs, suggesting that the principal passenger leukocytes were donor DCs which activated recipient T cells (Lechler and Batchelor 1982b, 1982a).

1.3.2 Indirect

The experiments performed by Lechler and Batchelor showed that depletion of donor DCs extended the survival rate of the rat kidney allografts. However, rejection was not completely avoidable, although it was at a slower kinetic in comparison to allografts that retained allogeneic DCs, this suggested that alloreactive T cells ought to be activated via a different pathway of allorecognition which would not implicate donor APCs (Lechler and Batchelor 1982b, 1982a). The genetic codes associated with antigen presentation contained within a MHC are the most polymorphic in vertebrates (Sommer 2005). Thus, it was conceivable that graft rejection is primarily activated by the recognition of donor MHC molecules presented in the intact arrangement (direct allorecognition) or as allopeptides presented in the context of recipient MHC molecules (indirect allorecognition). The frequency of T cells with indirect allospecificity is approximately 100-fold less than T cells with direct allospecificity (Z. Liu et al. 1993). Due to this large difference in frequency, it is thought that the indirect alloimmune responses are not as intense as the direct alloimmune response, although due to the constant supply of donor antigens in the allograft it marked the indirect pathway enabled to continue in extended terms. Thus, the indirect pathway is anticipated to be the chief player in chronic allograft rejection (Ali et al. 2013). The initial experiments

and report that demonstrated the involvement of the indirect pathway to allograft rejection was performed by Auchincloss *et al.* (Auchincloss et al. 1993). The authors transplanted skin grafts derived from a MHC class II knockout mice onto recipient mice which had $CD8^+$ T cells removed (thus these mice were dependent on $CD4^+$ T cell function) or $CD4^+$ T cells removed. The $CD8^+$ T cells- depleted mice rapidly rejected the MHC class II-deficient skin graft compared to the $CD4^+$ T cells- depleted mice. This demonstrated the important role of $CD4^+$ T cells in driving the allograft rejection process. Given that the donor grafts lacked MHC class II molecules, the $CD4^+$ T cells was thus primed by recipient APCs presenting allopeptides (Auchincloss et al. 1993). Subsequently, Lee *et al*. demonstrated that the $CD4^+$ T cells then helps activate $CD8^+$ T cells to further drive allograft rejection (Lee et al. 1994).

1.3.3 Semi direct

Cross-talk between $CD4^+$ and $CD8^+$ T cells during an immune response stipulates a 'three-cell' or 'linked' model in which both $CD4^+$ and $CD8^+$ T cells are activated by the same APCs (Ridge, Di Rosa, and Matzinger 1998). In this model, the $CD8^+$ T cells required help from $CD4^+$ T cells activated by the same APC. In the context of transplantation, cross-talk between the direct and indirect pathways of allorecognition would represent a conundrum to this model as it would require that direct pathway $CD8^+$ T cell and indirect pathway $CD4^+$ T cells are activated by donor APCs and recipient APCs, respectively, thus demanding a 'four-cell' or 'unlinked' model. This apparent paradox can be addressed by the semi-direct pathway of allorecognition (Herrera et al. 2004), in which the recipient APCs can acquire and present intact donor MHC-allopeptide complexes by MHC transfer mechanisms by direct cell to cell contact (Game, Rogers, and Lechler 2005) or via exosomes (Morelli et al. 2004), along with this is the presentation of allopeptides in the indirect manner (Herrera et al. 2004). Consequently, both $CD4^+$ and $CD8^+$ T cells can be activated by the same APC and thus linked help or linked suppression can be addressed by a 'three- cell' model.

The passenger leukocyte theory states that post-transplantation, donor APCs that reside in the allograft present donor MHC molecules directly to alloreactive T cells in secondary lymphoid organs. Although recently, Marino *et al.* (Marino et al. 2016) and

Liu *et al.* (Q. Liu et al. 2016) revisited this concept, these authors demonstrated that donor exosomes can transfer donor DC MHC molecules to recipient APCs, a phenomenon termed 'cross-dressing' via exosomes. These recipient APCs that acquired exosomes became activated and were able to activate alloreactive T cells (Marino et al. 2016; Q. Liu et al. 2016) and the donor exosomes rather than the passenger leukocytes initiate the alloresponse post-transplantation (Marino et al. 2016).

Recently, the host laboratory demonstrated in indirect pathway-deficient recipient mice (lacking cross priming/cross presentation) that acquired allo-MHC class I-peptide complexes on recipient DCs may be the main source of alloantigen that drives $CD8^+$ cytotoxic T cell responses and promote allograft rejection that can persist for the life-span on the transplant (L A Smyth, Lechler, and Lombardi 2017).

1.4 Immunosuppressive drugs

Undeniably, immunosuppressive drugs have improved allograft survival but disposes the patients more susceptible to other health complications such as increased risk of cancer and acquiring infections (Lizotti Cilião et al. 2016; Welzl et al. 2014). T cells are the main culprits in driving transplant rejection with B cells playing a role via the production of allo-antibodies (Ali et al. 2013). Transplant rejection can be hyper-acute, acute or chronic. Hyper-acute rejection can occur within minutes post-transplantation as a response to pre-formed antibodies against donor human leukocyte antigen (HLA) (human MHC) (Moreau et al. 2013). Acute rejection can occur from the first week to several months post-transplantation and mainly involves the direct pathway of allorecognition (Moreau et al. 2013). Chronic rejection can occur years post-transplantation and mainly involves the indirect pathway of allorecognition (Moreau et al. 2013). When allorecognition is initiated, a concerted course of activation of T cells leads to generation of effector T cells and subsequent production of various cytokines, allo-antibody production by activated B cells and macrophage activation altogether amplifies the alloimmune response. Over time, chronic rejection forces the graft to remodel its extracellular matrix caused by fibrotic scarring of expanding collagen fibres, disorganisation of fibronectin and proteoglycans which leads to progressively suboptimal functioning grafts, ultimately rendering graft failure (Moreau et al. 2013;

Rienstra et al. 2010; McManus et al. 1994). Modern immunosuppressants used to prevent graft rejection consists of various types of drugs aimed at targeting multiple cell types and their signalling pathways and suppressing the production of certain antibodies and cytokines. While the drugs used may vary between institutions, the treatment course will generally consist of a combination of calcineurin inhibitors (CNI), corticosteroids, anti-proliferatives and mammalian target of rapamycin (mTOR) inhibitors. At Guy's Hospital (London, UK), the standard set of drugs prescribed to kidney transplant patients consists of tacrolimus (a CNI inhibitor), prednisone (a corticosteroid) and mycophenolate mofetil (MMF; an anti-proliferative).

CNIs such as cyclosporine A and tacrolimus function by inhibiting the signalling phosphatase calcineurin, resulting in a block of T cell proliferation (Hamawy 2003). Due to CNIs' selective activity on T cells, use of these drugs permit the maintenance of other myeloid-derived cell types and reduces the doses of corticosteroid required (Haberal et al. 2004). Both of these CNIs inhibit the production of pro-inflammatory cytokines IL-2, IFNγ and TNFα despite having differential effects on IL-10 and TGFβ (Duncan and Wilkes 2005).

Prednisone is a common corticosteroid used in transplantation therapy, although they are not as selective in action compared to CNIs since they affect multiple leukocyte cell types including T cells, B cells, macrophages, granulocytes and monocytes. These steroid hormones function by inhibiting the activation of several pro-inflammatory genes in the abovementioned leukocytes (Duncan and Wilkes 2005). Corticosteroids are lipophilic and can permeate across cell membranes where they bind glucocorticoid receptors in the cytoplasm. In lymphocytes, glucocorticoids negatively regulate cytokine gene expression, inflammatory enzyme production and synthesis of inflammatory receptors by targeting and inhibiting pro-inflammatory transcription factors such as activator protein-1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Duncan and Wilkes 2005).

Anti-proliferative drugs such as MMF function by preventing *de novo* synthesis of purine, specifically preventing the synthesis of guanine thus inhibiting DNA and RNA production, which ultimately leads to prevention of T cell and B cell metabolism and their clonal expansion (Duncan and Wilkes 2005). Also, MMF functions by inhibiting antibody production which includes anti-HLA antibodies, reduces the proliferation of

cytotoxic natural killer (NK) cells and delayed-type hypersensitivity (DTH) response (Duncan and Wilkes 2005).

Rapamycin is an example of a mTOR inhibitor that is structurally related to tacrolimus and similar in that it binds the FK506-binding protein 12 (FKBP12), but instead of targeting the calcineurin pathway, the rapamycin-FKBP12 complex inhibit the mTOR pathway. This inhibition of mTOR results in prevention of the translation of messenger ribonucleic acid (mRNAs) that are associated with cell cycle progression from G1 to S phase, thus preventing T cell proliferation (Duncan and Wilkes 2005).

Despite these approaches, the half-life of a transplant is less than 10 years and one of the main aims of transplantation research is to induce a state of tolerance towards allogeneic antigens, thus reducing or eliminating the need for immunosuppression.

1.5 Mechanisms of tolerance

Immunological tolerance is a state of unresponsiveness of the immune system to antigens. Tolerance towards self-antigens is important to abrogate the development of various autoimmune diseases. Central tolerance is the tolerance achieved by deleting autoreactive cells before they can develop into fully incompetent cells. Peripheral tolerance occurs after T and B cells have matured and this process ensures that autoreactive cells which have escaped central tolerance do not initiate the development of autoimmune disease (Mueller 2010; Xing and Hogquist 2012).

1.5.1 Central tolerance

Central tolerance mechanisms occur in the primary lymphoid organs of the thymus and bone marrow for T cells and B cells, respectively. TCR and BCR genes contain multiple gene segments which undergoes a rearrangement process called variable-diversity-joining (V(D)J) recombination to generate a functional gene. V(D)J recombination occurs in common lymphoid progenitor (CLP) cells to generate TCR or BCR with a given specificity. The random nature of the process will likely generate T cells and B cells with a receptor that can recognise self-antigens with a high affinity.

Thymocytes are originally $CD4^-$ and $CD8^-$ (double negative (DN)), after which T cell lineage commitment and development, TCR gene arrangement occurs and produces αβ or γδ progenitors. When αβ processes continue within the DN cells, this produces $CD4^+$ and $CD8^+$ (double positive (DP)) thymocytes with somatic recombination of TCR genes which provides various αβ TCR with a wide range of specificity. The TCR affinity for self-peptide-MHC complexes governs the fate of the thymocytes at this point (Klein et al. 2014). DP thymocytes that do not recognise self-peptide MHC complexes undergo programmed cell death; whereas, if they recognise and bind self-peptide MHC complexes with a high affinity, they will undergo negative selection, thus preventing autoimmune conditions. Hence, the remaining DP thymocytes would be cells that bind to self-peptide MHC complexes with a low affinity and these cells are positively selected to differentiate into $CD4^+$ or $CD8^+$ (single positive (SP)) thymocytes and released into the periphery as naïve T cells. Furthermore, of the $CD4^+$ SP thymocytes compartment, the cells that binds self-peptide-MHC complexes with a higher than average affinity will undergo an induction process to preferentially express Forkhead box P3 (FoxP3) and commit to the Treg lineage.

1.5.2 Peripheral tolerance

Although the central tolerance mechanisms are thorough, there are some autoreactive cells that may still escape this processing and arrive into the periphery. The potentially harmful impacts of these cells are managed by peripheral tolerance mechanisms which occur mainly in the lymph nodes. These peripheral tolerance mechanisms are described as below.

1.5.2.1 Peripheral deletion by apoptosis

Following T cell activation and expansion due to the initiation of a target antigen and eliminating the threat, T cell numbers have to be declined to ensure a return to normal immune cell homeostasis. Activation-induced cell death (AICD) is a natural capability of T cells to regulate their frequencies (Waring and Müllbacher 1999). T cells express Fas (cluster of differentiation (CD)95) and post TCR engagement upregulate Fas Ligand (FasL) expression (J. Zhang, Xu, and Liu 2004). Thus, T cells can induce apoptosis in an

autocrine manner via the Fas/FasL pathway (Dhein et al. 1995). Watanabe-Fukunaga *et al*. (Watanabe-Fukunaga et al. 1992) and Takakasi *et al.* (T. Takahashi et al. 1994) showed that Fas and FasL deficient mice exhibit signs of defects in peripheral tolerance by abnormal inflammation and autoimmune disease development. Marrack and Kappler (Marrack and Kappler 2004) and Mueller (Mueller 2010) demonstrated that Bcl-2-like protein 11 (Bim) and B-cell lymphoma 2 (Bcl-2)/ B-cell lymphoma-extra large (Bcl-xL) deficient mice also are unable to clear autoreactive lymphocytes leading to autoimmune disease in these mouse models.

1.5.2.2 Immunological ignorance

'Immunoprivileged organs' are organs whereby the antigens are tolerated without mounting an inflammatory immune response. Such organs include the brain, eyes, placenta, foetus and testicles. Anatomical barriers can physically separate the autoreactive T cells from reaching the antigen, for example within the central nervous system of the blood brain barrier, and thus autoreactive T cells are not activated in these anatomical sites.

1.5.2.3 T cell anergy and costimulatory signals

T cell activation principally require TCR engagement, however, to prompt an immune response a secondary co-stimulatory signal is required. During the first signal, the TCR recognises and binds to a cognate MHC-peptide complex. During the second signal, the co-stimulation can be provided by several different co-stimulatory molecules. The main co-stimulation is provided by CD28 which interacts with CD80/CD86 co-stimulatory molecules expressed on APCs; thus, subsequently promoting T cell survival, activation and proliferation (Lieping Chen and Flies 2013). Activation of T cells without CD28 results in an anergic or unresponsive state which may lead to their deletion. Other co-stimulatory molecules include CD27, tumour necrosis factor receptor superfamily member 4 (OX40), tumour necrosis factor receptor superfamily member 9 (41BB), inducible T cell costimulatory (ICOS) and CD40 ligand (CD40L), which its relative ligands are found on the surfaces of APCs (Lieping Chen and Flies 2013). Noteworthy, within the

peripheral system, autoreactive T cells can also be controlled by regulatory immune cells.

1.6 Regulatory T cells

In 1969, Nishizuka and Sakakura were the first to experimentally demonstrate that thymocytes have regulatory properties and they suggested that this contributed to the prevention of autoimmune disease (Nishizuka and Sakakura 1969). In 1970, Gershon and Kondo made the important discovery that certain T cells that were distinct from helper T cells can inhibit the immune responses (Gershon and Kondo 1970). In 1972, their research gained considerable attention after the publication of their article which coined the term 'suppressor T cells' and how T cells can inhibit the immune response (Gershon et al. 1972). These findings spurred many immunologists to research 'suppressor T cells' but their interest sharply collapsed in the mid-1980s when the molecular analysis of the mouse MHC gene revealed no existence of the I-J molecule which was supposedly a key suppressor molecule directly linked with their suppressive function (Kronenberg et al. 1983). This halt in 'suppressor T cell' research was further compounded by the failure to identify a molecular marker to distinguish these 'suppressor T cells' compared to other T cells types along with the ambiguity in their mechanisms of suppressive action (Kronenberg et al. 1983).

It was not until 25 years later, in 1995, that Sakaguchi *et al.* published a seminal article demonstrating these 'suppressor T cells' can be distinguished from other $CD4^+$ T cells by the expression of interleukin (IL)-2 receptor alpha chain (CD25) molecules and coined the term 'regulatory T cells (Tregs)' (S Sakaguchi et al. 1995). Sakaguchi *et al.* showed that the depletion of these $CD4^+CD25^+$ T cells resulted in various autoimmune diseases whereas the adoptive transfer of these cells into day 3 thymectomised mice prevented the autoimmune diseases (S Sakaguchi et al. 1995).

Following this discovery, many groups published concurrently in 2001 demonstrating the existence of human $CD4^+CD25^+$ Tregs and their properties (Ng et al. 2001; C Baecher-Allan et al. 2001; Taams et al. 2001; Stephens et al. 2001; H Jonuleit et al. 2001; Dieckmann et al. 2001; M K Levings, Sangregorio, and Roncarolo 2001).

1.6.1 Treg subtypes

1.6.1.1 $CD4^+$ regulatory T cell subtypes

Even though the field of Treg research is ever expanding in knowledge, there are controversies as to what defines a Treg. Tregs are not a single homogenous cell type but consists of many subpopulations, which can be distinguished by their surface markers and/or their expression levels of these markers, their mechanisms of activation and what molecular tools they use to suppress the target cells. Furthermore, although mouse and human Tregs do have some fundamental similarities there are disparities that exist, which will be explained below.

The consensus and at the most basic level there are two main categories of $CD4^+$ Tregs: $CD4^+$ Tregs that are derived from the thymus (tTregs) and $CD4^+$ T cells which are induced either in the periphery (pTregs) or *in vitro* (iTregs) to become suppressive. Other types of regulatory T cells also exist which are $CD4^-CD8^+$ Tregs and $CD4^-CD8^-$ Tregs, but these are less well-defined as the $CD4^+$ Tregs.

1.6.1.2 Thymus-derived $CD4^+$ Tregs

Thymus-derived Tregs were previously termed 'naturally-occurring Tregs' and are $CD4^+$, $CD25^+$ and $FoxP3^+$ (Romano et al. 2017). These Tregs constitutes approximately 5-10% of total peripheral $CD4^+$ T cells (H Jonuleit et al. 2001) and 1-2% of whole blood. They are positively selected in the cortex via TCR through high-affinity recognition with self-peptides-MHC complexes presented by thymic stromal cells. Through this process, these cells become anergic and are resistant to negative selection (Maggi et al. 2005). Tregs require activation of their TCR to become suppressive, but once activated they can suppress in an antigen independent manner (A M Thornton and Shevach 2000).

tTregs constitutively express the lineage-determining transcription factor FoxP3 (Romano et al. 2017) and in the absence of functional FoxP3, $CD4^+CD25^+$ Treg cells do not develop (van der Vliet and Nieuwenhuis 2007). Many groups have demonstrated that FoxP3 is a key player in immune suppression (S. Sakaguchi et al. 2009; Bin Dhuban et al. 2017; Kim 2009; Mercer and Unutmaz 2009) and its loss of FoxP3 expression levels in cells are linked to their reduced ability to suppress (Romano et al. 2017). The importance of the FoxP3 gene has been shown by genetic mutation resulting in mice

displaying a “scurfy” phenotype which is characterised by a scaly/ruffled skin, red irritated eyes, enlargement of secondary lymphoid organs and an early onset of death (Ramsdell and Ziegler 2014). In humans, FoxP3 mutations results in immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome disorder which is characterised by a variety of inflammatory conditions such as dermatitis, cachexia, thyroiditis, autoimmune haemolytic anaemia and membranous nephropathy (Wildin, Smyk-Pearson, and Filipovich 2002; Torgerson 2006) and if left untreated, children do not tend to live beyond 2 years of life due to sepsis and other complications (van der Vliet and Nieuwenhuis 2007).

Currently, the only way to distinguish tTregs is the evaluation of the Treg-specific demethylated region (TSDR), an evolutionarily conserved noncoding element within the FoxP3 gene locus, which is fully demethylated in tTregs (Toker et al. 2013). Whilst in the mouse the expression of neuropilin-1 has helped in distinguishing between tTregs and pTregs (Weiss et al. 2012; Yadav et al. 2012) in human, this is not possible (Milpied et al. 2009).

1.6.1.3 Periphery-derived and *in vitro*-induced $CD4^+$ Tregs

pTregs and iTregs are altered states of differentiation of conventional $CD4^+$ T cells which their suppressive ability can be temporary and thus controversially in the T cell field, it is sometimes not defined as a unique cell lineage per se (Helmut Jonuleit and Schmitt 2003) , but rather a ‘phase’.

pTregs and iTregs are Tregs that are induced to be suppressive but are different in that pTregs are derived from *in vivo* conditions, whereas iTregs are derived from *ex vivo* conditions. Among pTregs and iTregs, arising from conventional $CD4^+CD25^-$ T cells (Tconv) in the periphery under specific conditions, are the T helper (Th) 3 cells and the type-1 regulatory T cell (Tr1). The presence of transforming growth factor beta 1 (TGF-β) and IL-4 promotes the induction of Th3 cells which in turn predominately secretes immunosuppressive TGF-β (Weiner 2001), whereas the presence of IL-10 and interferon gamma (IFNγ) induces Tr1 cells which predominantly secretes IL-10 into the microenvironment (Roncarolo et al. 2014; Grazia Roncarolo et al. 2006).

Another type of pTregs are the induced $CD4^+CD25^+FoxP3^+$ pTregs which are generated from peripheral $CD4^+FoxP3^-$ T cells upon activation and in the presence of TGF-β and IL-2 (Kanamori et al. 2016); these Tregs display similar cell surface markers as tTregs and function by contact-dependent mechanisms and the release of immunosuppressive cytokines. TSDR methylation status is a key to distinguishing between the thymus-derived $CD4^+CD25^+FoxP3^+$ and the peripheral-derived $CD4^+CD25^+FoxP3^+$ Tregs.

Both Tr1 and Th3 cells are principally associated with mucosal immunity since Tr1 cells have been used to therapeutically treat Crohn's disease (Desreumaux et al. 2012), however due to these cells inherent instability it questions the potential of these cells in clinical treatments (A M Thornton and Shevach 2000; Q. Chen et al. 2011).

As abovementioned, whilst in the mouse the expression of neuropilin-1 has helped in distinguishing between tTregs and pTregs (Weiss et al. 2012; Yadav et al. 2012) in human, this is not possible (Milpied et al. 2009). However, it has been suggested from mouse and human studies that Helios expression may be able to differentiate between tTregs which are $Helios^+$ and pTregs which are $Helios^-$ (A. K. Singh and McGuirk 2016; Fabricius and Ramanathan 2016). Moreover, the evaluation of TSDR methylation status can only be a tool in diagnosis or clinical trial monitoring but not used for Treg isolation, given that cells will have to be permeablised for this method of identification.

In 2009, Miyara *et al*. demonstrated that human Tregs in peripheral blood are heterogeneous and consists of three main subpopulations based on their diverse functional ability and their differential expression levels of FoxP3 and CD45RA (Miyara et al. 2009). Tregs can be divided into (i) naïve/resting and very stable cells expressing $CD45RA^+FoxP3^{low}$; (ii) effector Tregs expressing $CD45RA^-FoxP3^{high}$; and (iii) cytokine-producing Tregs, expressing $CD45RA^-FoxP3^{low}$.

The naïve/resting Treg population are considered the 'real Tregs'; they are potently suppressive and fully demethylated in the FoxP3 locus (Romano et al. 2017). Under *in vitro* conditions, the naïve/resting Treg population proliferates whereas the effector Treg population die whilst exerting suppression. Although Sakaguchi's group suggested that the cytokine-secreting $CD45RA^-FoxP3^{low}$ population was 'non-suppressive' (Miyara et al. 2009) there is sufficient evidence to suggest that this population of Tregs is

suppressive (Afzali et al. 2013). Furthermore, this CD45RA^{-}FoxP3^{low} population contain cells with Th17 cell potential (Miyara et al. 2009; Scottà et al. 2013; Afzali et al. 2013).

1.6.1.4 CD8^{+} Tregs

As previously mentioned, CD8^{+} Tregs are not as well-characterised as CD4^{+} Tregs. Nonetheless, CD8^{+} Tregs are similar to their CD4^{+} counterparts in that they can be derived naturally or induced (Pomié, Ménager-Marcq, and van Meerwijk 2008). There are 4 main types of CD8^{+} Tregs: mouse and human CD8^{+}CD25^{+}FoxP3^{+}CD28^{-} T cells (Churlaud et al. 2015; Najafian et al. 2003), mouse CD8^{+}Qa-1^{+} T cells (Hu et al. 2004), mouse CD8^{+} CD122^{+}CD49^{low} T cells (Akane et al. 2016) and rat CD8^{+}CD45RC^{low} T cells (Xystrakis et al. 2004). CD8^{+} Tregs have been shown in animal models to be efficient in preventing experimental autoimmune encephalomyelitis (EAE) (Sinha, Itani, and Karandikar 2014) and colitis principally driven by their role of secreting potent immunosuppressive cytokines (Endharti et al. 2011). Bezie *et al.* showed that rodent and human CD8^{+} Tregs produce IL-34, and this cytokine promoted transplant tolerance by inhibiting allo-antibody production in rat cardiac allograft models (Bézie et al. 2015). Taken together, these studies compliment other studies which demonstrated that in human subjects, low frequencies of CD8^{+} Tregs were associated with a higher incidence of systemic erythematosus (SLE) (Filaci et al. 2001; Tulunay et al. 2008), inflammatory bowel disease (Brimnes et al. 2005) and multiple sclerosis (MS) (Frisullo et al. 2010). Thus, although CD8^{+} Tregs are less well-characterised in literature compared to CD4^{+} Tregs, it is evident that CD8^{+} Tregs contributes to the prevention of autoimmune diseases.

For this thesis, all the 'Tregs' referred from here onwards will be the CD4^{+} Tregs unless otherwise stated.

1.6.2 Phenotypical characterisation of Tregs in mouse and human

Since the discovery of Tregs, their phenotypical characterisation has been elucidated to some extent, although to date, there is not a unique definitive marker to distinguish them from other types of T cells. Accordingly, Tregs are often described using a

combination of different cell markers as outlined below. Some of these cell markers play a role in Treg mechanisms of suppression which are further discussed below.

1.6.2.1 CD25

As abovementioned, Sakaguchi *et al.* discovered CD25 expression as an important marker of mouse Tregs (S Sakaguchi et al. 1995). CD25 is the IL-2 receptor α chain, which is part of the IL-2 receptor (IL-2R) complex with the β and γ chains which together provides a high affinity for IL-2. Given that IL-2 is a T cell growth factor and is essential for their survival and clonal expansion, it is transiently upregulated on activated conventional human T cells (Kmieciak et al. 2009), rendering this marker not exclusive to Tregs. However, in human conventional T cells, the CD25 expression is of intermediate levels compared to human Tregs where they have high expression of CD25, thus the $CD25^{hi}$ expression can be used alongside other characterisation criteria to diverge the two populations.

1.6.2.2 FoxP3

It was not until 2003, the transcriptional regulator FoxP3 was discovered as a master control gene for mouse Tregs and their expression is important for mouse Treg definition (Fontenot, Gavin, and Rudensky 2003; S. Hori, Nomura, and Sakaguchi 2003; Khattri et al. 2003). In the mouse it is present exclusively on Tregs and thus it is an appropriate marker to define them. However, in human, FoxP3 expression is not exclusive to human Tregs given that human $CD4^{+}CD25^{-}$ conventional T cells transiently upregulate FoxP3 expression following their activation (Kmieciak et al. 2009). As explained above, one of the ways to identify bona fide Tregs is the evaluation of the TSDR (Romano et al. 2017), which is fully demethylated in Tregs but methylated in conventional T cells. Tregs can maintain a stable expression of FoxP3 via the epigenetic modification of the TSDR, which is an evolutionarily conserved non-coding element of the FoxP3 gene locus (Schreiber et al. 2014).

1.6.2.3 CD127

Although TSDR analysis and FoxP3 expression are relatively reliable methods of identifying Tregs, it does require cells to be lysed or permeablised, respectively, rendering this method of Treg identification as an *in vitro* analysis rather than employed as a means of isolation of Tregs. Thus, the identification of cell surface marker(s) that are differentially expressed by Tregs and activated conventional T cells are essential to separate these two populations of cells. CD127 is the IL-7 receptor α chain found on the cell surface which is highly expressed by conventional T cells but Tregs have a low to negative expression of this molecule (W. Liu et al. 2006). In 2006, *Lui et al.* discovered that CD127 expression is inversely correlated with FoxP3 expression (W. Liu et al. 2006) thus CD127 is so far the best marker to distinguish and isolate human Tregs in combination with $CD4^+$ and $CD25^{high}$ expression (Romano et al. 2017). Indeed, cell sorting using the criteria of $CD4^+CD25^{hi}$ and $CD127^{lo}$ for human Tregs is of current practice for the isolation of pure Tregs.

1.6.2.4 CTLA-4

Cytotoxic T lymphocyte associated protein 4 (CTLA-4) is an inhibitory protein related to the T cell costimulatory molecule CD28. Signalling through CD28 promotes T cell activation whilst signalling through CTLA-4 suppresses T cell activation. Despite their contrasting functions, both molecules engage with the same ligands of CD80 and CD86. CTLA-4 uses a diverse array of mechanisms to suppress target cells. CTLA-4, which is constitutively expressed on Tregs, is an indispensable regulator of peripheral tolerance, however it is not exclusive to Tregs as it is also found present on activated conventional T cells (Tai et al. 2012). CTLA-4 is predominately an intracellular protein and thus like for FoxP3 Tregs need to be permeablised, rendering CTLA-4 expression helpful for *in vitro* analysis of Tregs but not for their isolation.

1.6.2.5 Helios

Helios is an Ikaros transcription factor family member which its expression is present in 100% of $CD4^+CD8^-FoxP3^+$ thymocytes but in peripheral lymphoid tissues of mouse and human Tregs it is present in only 70% of $FoxP3^+$ Tregs (Angela M Thornton et al. 2010).

Nevertheless, Thornton *et al.* described that Helios expression can be used to differentiate tTregs from pTregs (Angela M Thornton et al. 2010). However, subsequent studies indicated that this is not possible as Helios could be induced in pTregs (Gottschalk, Corse, and Allison 2012; Akimova et al. 2011) and that Helios$^+$ and Helios$^-$ Tregs are present in the repertoire of tTregs (Elkord 2016; Akimova et al. 2011). Despite this, human FoxP3$^+$Helios$^+$ Tregs are more suppressive compared with FoxP3$^+$Helios$^-$ Tregs (Elkord, Samid, and Chaudhary 2015), and Helios can work cooperatively with FoxP3 to enhance Treg function (Takatori et al. 2015).

1.6.2.6 CD45RA and CD45RB

CD45RA is an isoform of CD45 which is found mainly expressed on naïve T cells. After TCR activation, when a naïve T cell becomes a memory T cell, CD45RA expression is downregulated whilst CD45RO is upregulated. Thus, naïve T cells are defined as CD45RA$^+$CD45RO$^-$ and memory/activated T cells are defined as CD45RA$^-$CD45RO$^+$ (Machura et al. 2008).

As described above, Miyara's findings showed that CD45RA$^+$ Tregs constitutively express FoxP3 and CD25 (Miyara et al. 2009). Given the complication of activated conventional T cells upregulating FoxP3 and CD25 expression following their activation (Kmieciak et al. 2009), CD45RA and CD45RO expression can differentiate between naïve Tregs from activated conventional T cells, thus facilitating 'pure' Treg identification and isolation. However, in adults approximately 80% of tTregs are CD45RO$^+$, with the population of CD45RA$^+$ Tregs decreasing with age. The CD45RO$^+$ Tregs are more highly proliferative compared to the CD45RA$^-$ Tregs, which was likely due to the Treg activated status (N. J. Booth et al. 2010). There is a subpopulation of Tregs which express low or high levels of CD45RB. The expression of CD45RB can further define the memory cell populations (Horgan et al. 1994).

1.6.2.7 CD39 and CD73

CD39 and CD73 are both cell surface bound enzymes (ectoenyzmes) that are found on human and mouse Treg cells. These ectoenzymes work in unison to promote an anti-

inflammatory microenvironment (Antonioli et al. 2013). The function of CD39 and CD73 will be further discussed in section 1.6.3.1.

1.6.2.8 Co-stimulatory and inhibitory receptors

Treg activation and expansion requires co-stimulatory signals. Members of the tumour necrosis factor (TNF) receptor (TNFR) superfamily such as glucocorticoid-induced TNFR-related protein (GITR) are present on Tregs. Furthermore, the majority of freshly isolated Tregs express high levels of GITR (Horgan et al. 1994; McHugh et al. 2002), whilst in resting conventional T cells they express low levels, but its expression is upregulated after activation (Ephrem et al. 2013).

Other members of the TNFR superfamily such as OX40, TNFR2 and CD27 are also present on Tregs, although these can also be upregulated in conventional T cells upon activation. Vu *et al.* demonstrated that stimulating OX40 on FoxP3$^+$ Tregs resulted in these Tregs reducing their ability to suppress T effector cell (Teffs) proliferation, IFNγ secretion and Teff-mediated transplant rejection (Vu et al. 2007).

Koenen *et al.* demonstrated that the CD4$^+$ Tregs subpopulations of CD27$^+$ and CD27$^-$ although are both highly suppressive, the CD27$^+$ Treg subpopulation was able to suppress ongoing T cell responses (Hans J P M Koenen, Fasse, and Joosten 2005), thus CD27$^+$ expressing Tregs could be a favourable Treg subset in terms of suppressive potency compared to CD27$^-$ Tregs.

Other markers have been used to identify various subpopulations of Tregs such as the expression of the co-inhibitory molecules programmed cell death protein 1 (PD-1), the expression and function of lymphocyte-activation gene 3 (Lag-3) and the expression of HLA class II molecule HLA-DR.

1.6.2.9 Homing receptors

The expression of homing receptors is important for Tregs to traffic and home to certain areas *in vivo*. Homing receptors can also be used to identify Tregs and their subpopulations. For example, human naïve CD45RA$^+$ Tregs express various lymphoid homing receptors including L-selection (CD62L) and chemokine receptor (CCR)-7 which

both allow entry into lymph nodes and C-X-C chemokine receptor type 4 (CXCR4) (P. Hoffmann et al. 2006). Whereas, for memory T cells there are two subsets with distinct homing receptor expression patterns and different effector functions. The $CCR7^-$ memory T cells express markers for migrating to the site of inflamed tissues and exert immediate effector function, whereas the $CCR7^+$ memory T cells express homing receptors to migrate to the lymph nodes but do not exert immediate effector function (Sallusto et al. 1999).

1.6.3 Treg suppression mechanisms

Tregs employ a plethora of contact dependent and independent mechanisms to exert their suppressive function on different target cells such as $CD4^+$ and $CD8^+$ T cells, macrophages, DCs, NK cells and B cells to maintain immune tolerance. Thornton and Shevach demonstrated that Tregs require TCR stimulation to suppress in an antigen non-specific manner (A M Thornton and Shevach 2000). Novel mechanisms of Treg suppression are still being discovered, thus Treg suppression mechanisms and their biology is not fully elucidated. It is not clear which suppression mechanism(s) pre-dominates during an immune response, but it could be conceived that an arsenal of these mechanisms may be employed depending on the target cell or the microenvironment it is in.

The various suppressive mechanisms used by Tregs could be broadly divided into five main 'modes of action'; metabolic interference; inhibitory cytokine release; cytolysis; targeting APCs/inhibition of maturation (A M Thornton and Shevach 2000) and more recently the release of inhibitory extracellular vesicles (Tung et al. 2018; Lesley Ann Smyth et al. 2013; X. Yu et al. 2013; Aiello et al. 2017; Azimi et al. 2018; Torri et al. 2017; Okoye et al. 2014) (Figure 1.2).

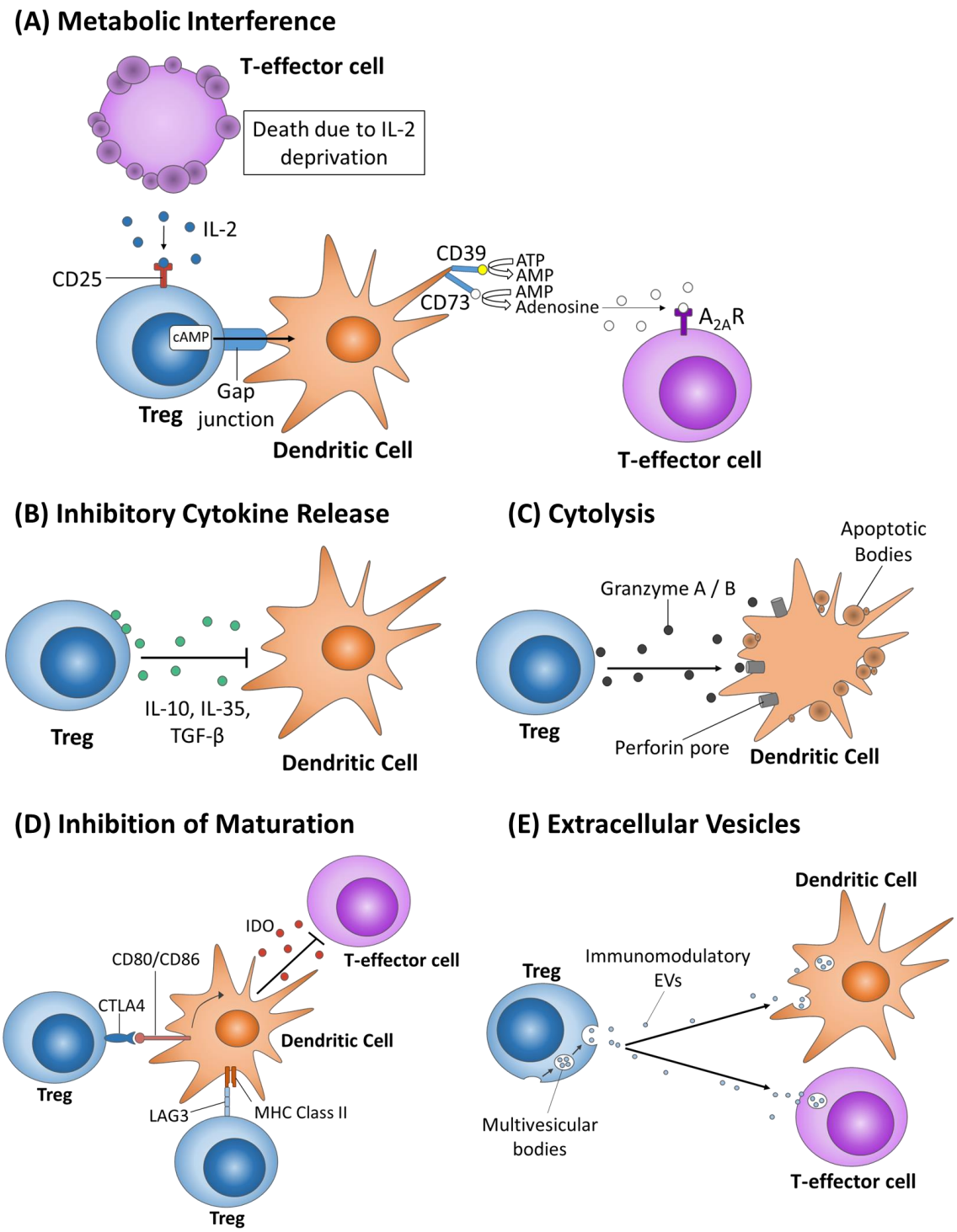


Figure 1.2- Treg suppression mechanisms

Schematic diagrams illustrating the various Treg suppression mechanisms. ***(A)*** *Metabolic interference* ***(B)*** *Inhibitory cytokine release* ***(C)*** *Cytolysis* ***(D)*** *Inhibition of maturation and* ***(E)*** *Extracellular Vesicles*

1.6.3.1 Metabolic interference

Teffs depend on IL-2 for survival and proliferation; Tregs constitutively express high levels of CD25, thus allowing Tregs to rapidly deplete and consume IL-2 from the microenvironment and thus limiting its availability for Teffs survival and function (S Sakaguchi et al. 1995). While earlier reports have suggested that this was not a bona fide Treg suppression mechanism (Fontenot et al. 2005; Duthoit et al. 2005), another report has suggested that Tregs may be involved in cytokine (in particular IL-2) deprived-mediated apoptosis (Pandiyan et al. 2007).

Additionally, the co-operative function of CD39 and CD73 ectoenzymes can together facilitate the conversion of pro-inflammatory adenosine triphosphate (ATP) into anti-inflammatory adenosine. Firstly, CD39 hydrolyses ATP/adenosine diphosphate (ADP) into adenosine monophosphate (AMP); secondly, CD73 catalyses the conversion of AMP into adenosine (Antonioli et al. 2013) which suppressed Teffs function through activating the A_{2a} receptor (Deaglio et al. 2007; Borsellino et al. 2007; Kobie et al. 2006). Furthermore, the linkage of adenosine to the A_{2a} receptor also inhibited IL-6 expression whilst increasing the production levels of TGF-β which promoted the induction of adaptive Tregs (Zarek et al. 2008).

Tregs can communicate with Teffs via gap junctions; these are membrane-bound channels that connect between cells to allow exchange of biological material. Tregs can harbour high amounts of cyclic adenosine monophosphate (cAMP), which is a second messenger that potently inhibits the synthesis of IL-2 and inhibits the proliferation of Teffs; Tregs can transfer cAMP into target cells via gap junctions (Bopp et al. 2007) as another means of metabolic interference. In addition, Tregs have the potential to deprive target cells of their energy sources via the induction of indoleamine 2, 3-dioxygenase (IDO) production (Yan et al. 2010). IDO is an enzyme that catalyses the degradation of tryptophan through the kynurenine pathway, which depletes tryptophan from the microenvironment leading to limiting energy resources/amino acids for Teffs growth and expansion (Yan et al. 2010).

1.6.3.2 Inhibitory cytokine release

Tregs can release immunosuppressive cytokines such as IL-10, IL-35 and TGF-β to prevent T cell proliferation and maturation of APCs (Chaudhry et al. 2011; Sawant,

Hamilton, and Vignali 2015; Wan and Flavell 2007). Asseman *et al.* showed that the transfer of CD45RB^{high} T cells induces intestinal inflammation but can be prevented with the addition of CD45RB^{low} T cells. These CD45RB^{low} T cells' suppressive ability was dependent on IL-10, as IL-10 deficient CD45RB^{low} T cells did not prevent colitis (Asseman et al. 1999). Other *in vivo* studies on infection and EAE models have also shown the importance of IL-10 for Treg function (Belkaid 2007; McGeachy and Anderton 2005). Rudensky's group used mice with conditional knock out of IL-10 gene in the FoxP3 gene locus induced by Cre recombinase to test the importance of IL-10 activity by Tregs. The authors showed that ablation of IL-10 does not affect Treg development nor does it cause systemic autoimmunity. However, they did show that IL-10 produced by Tregs was important for maintaining immunity at the environmental interfaces within the colon and lungs (Rubtsov et al. 2008).

Human and mouse Tregs are able to produce high levels of both membrane-bound and soluble TGF-β. Blocking TGF-β reduced the suppressive potency of Tregs towards T cell proliferation (K Nakamura, Kitani, and Strober 2001; Kazuhiko Nakamura et al. 2004; Megan K Levings et al. 2002). On the contrary, some groups have shown that TGF-β is not involved in Treg-mediated suppression of T cells (Clare Baecher-Allan, Viglietta, and Hafler 2002; Piccirillo et al. 2002; Godfrey et al. 2005; Oberle et al. 2007). However, this could be due to the different starting cell populations and purity used for the experiments and the different experimental conditions.

1.6.3.3 Cytolysis

Human Tregs express granzyme A and kill CD4$^+$ T cells in a perforin-dependent cytotoxic manner (Grossman et al. 2004). Whereas murine Tregs killing is partially granzyme B-dependent without the requirement of perforin (Noelle David C Gondek, Lu, and Quezada 2018). Tregs also express death ligands TNF-related apoptosis-inducing ligand (TRAIL) (Ren et al. 2007) and FasL (Strauss, Bergmann, and Whiteside 2009), which upon interaction with target cell death receptor expressed by CD8$^+$ T cells initiate a cascade of signalling events to induce caspase-related cell death. Galectin-9, which binds to T cell immunoglobulin and mucin-domain containing-3 (TIM-3) found on Teffs, led to caspase-related cell death is also expressed on Tregs (F. Wang et al. 2009).

1.6.3.4 Targeting of APCs

Tregs are the only T cell subpopulation that constitutively expresses CTLA-4; it binds CD80/CD86, the co-stimulatory molecules expressed by APCs, to block their binding to CD28, thus limiting T cell activation. Furthermore, CTLA-4 can also downregulate DCs' activity via trans-endocytosis or extraction of CD80 and CD86 resulting in diminished co-stimulation (Qureshi et al. 2011). Recently, in 2017 Mavin *et al.* demonstrated that human Tregs mediate transcriptional modulation of DCs (Mavin et al. 2017). The authors showed that DCs that had been treated with Tregs caused a semimature phenotype and morphology and further assessed their gene expression. Given that NF-kB signalling pathway plays an important role in DC maturation, the authors further investigated NF-kB-signalling related genes. Wnt5a was found to be higher expressed by DCs that had been treated with Tregs compared to mature DCs. The authors showed that in the absence of a Wnt5a inhibitor, DCs which were treated with Tregs showed a decreased surface expression of maturation and costimulation markers; CD38, CD83, CD80 and CD86 compared to mature DCs. This was partially reversed in the presence of the Wnt5a inhibitor. Thus, regulation of Wnt5a is a possible molecular tool used by Tregs to modulate DC reprogramming during their maturation process (Mavin et al. 2017).

1.6.3.5 Extracellular vesicles

Recently, a novel mechanism of Treg suppression was discovered by us and others (Tung et al. 2018; X. Yu et al. 2013; Lesley Ann Smyth et al. 2013; Aiello et al. 2017; Azimi et al. 2018; Torri et al. 2017; Okoye et al. 2014). It refers to the release of nano-sized extracellular vesicles (EVs), including exosomes, that are immunomodulatory. Treg-derived EVs inhibited target cell functions *in vitro* (Tung et al. 2018; Lesley Ann Smyth et al. 2013; X. Yu et al. 2013; Aiello et al. 2017; Okoye et al. 2014; Torri et al. 2017; Azimi et al. 2018) and functioned *in vivo* (Okoye et al. 2014; X. Yu et al. 2013; Aiello et al. 2017) to provide immune regulation.

1.6.4 Tregs in the clinic

1.6.4.1 Source of Tregs and their isolation

Due to the suppressive characteristic of Tregs, different laboratories have considered using Tregs in the clinic to treat various autoimmune diseases, prevent graft versus host disease (GvHD) and prevent solid organ transplant rejection. Thus, the need for Tregs isolated in a good manufacturing practice (GMP) manner that can be used for patients was required. The majority of preclinical Treg studies source the Tregs from peripheral blood or umbilical cord blood (Romano et al. 2017). A seminal report by Hoffman *et al.* demonstrated a GMP procedure for the isolation of $CD4^+$ $CD25^+$ T cells from standard leukapheresis products. The isolation of these cells was performed using a CliniMACS system which involves a clinical-grade magnetic enrichment of cells processed within a sterile and closed machine. This procedure consisted of two main steps; the first step involved depletion of $CD19^+$ cells and the second step involved enrichment of cells expressing CD25 molecules (Petra Hoffmann et al. 2006).

An alternative strategy to isolate Tregs is the flow-cytometry-based purification approach (Romano et al. 2017). The best marker to distinguish and isolate Tregs in combination with CD4 and CD25 is CD127 (W. Liu et al. 2006); its expression inversely correlates with FoxP3 and suppressive Treg function (W. Liu et al. 2006).

1.6.4.2 Treg expansion

From animal studies it was observed that there is a difference between freshly isolated and cultured mouse tTregs in their suppressive ability as shown by Thornton and Shevach (A M Thornton and Shevach 2000). Thornton and Shevach demonstrated that cultured tTregs can suppress the stimulation of $CD4^+CD25^-$ T cells 4- to 6-fold more efficiently in comparison to freshly isolated tTregs (A M Thornton and Shevach 2000), this suggests that *in vitro* expansion and culturing conditions can increase the suppressive potency of these cells.

Considering the relatively low number of Tregs present in peripheral blood and umbilical cord blood, the infusion of large numbers of freshly isolated Tregs is difficult to achieve. Therefore, to attain a higher number of Treg cells for infusion into patients in the treatment of both GvHD and solid organ transplantation, Tregs have been

expanded *ex vivo* using anti-CD3/CD28-coated beads along with high doses of IL-2 (Romano et al. 2017). The host laboratory has finished two clinical trials in kidney and liver transplant patients using expanded Tregs.

A potential issue of Treg isolation using the immunomagnetic approach is that the resultant cells can be contaminated with Teffs. The host laboratory has developed Treg expansion protocols using rapamycin and all-trans retinoic acid (ATRA) to minimise the contaminating Teffs population whilst favouring the expansion of Tregs. Rapamycin is an mTOR inhibitor which significantly decreases the undesired expansion of Teffs whilst permitting Tregs to proliferate due to their independency of the mTOR pathway for cell cycle progression (Thomson, Turnquist, and Raimondi 2009). Furthermore, rapamycin confers to the expanded Tregs a higher stability and suppressive capacity *in vitro* and in GvHD mouse models (Romano et al. 2017). ATRA affects T cell fate by contributing to Treg differentiation in the presence with TGF-β, however the effect of ATRA on tTregs remain controversial and therefore not currently used for GMP Treg expansion protocols. Tregs can be skewed to express certain homing receptors by culturing them in the presence of specific drugs. The host lab has previously published a report describing how rapamycin and ATRA treatment on Tregs can confer them with specific homing receptor expression patterns. Namely, rapamycin treatment on Tregs induced the co-expression of skin homing receptors CCR4 and cutaneous lymphocyte-associated antigen (CLA), whereas the ATRA treatment induced the co-expression of liver and gut homing receptors CCR9 and α4β7 integrin, respectively. The dual treatment of rapamycin and ATRA resulted in expression of a combination of homing receptors featured by the treatment of each individual drug (Scottà et al. 2013). Despite the success and the demonstration so far that Tregs are safe there is always a risk that Tregs once injected *in vivo* are not stable, as they have been shown to be plastic (Shimon Sakaguchi et al. 2013; Shohei Hori 2014; Beyer and Schultze 2011).

1.6.5 Treg plasticity

Several studies have suggested that inflammatory environments can subvert human FoxP3^{+} Treg function by converting them to Teffs *in vivo* (Zhou et al. 2009; Waldmann et al. 2014). Tregs are used in various clinical trials, however, how Tregs function *in vivo* in

humans, the longevity of Tregs and the trafficking of Tregs are still not completely understood. But given the fact that the usage of Tregs may enter the clinical arena as a potential standard therapy for transplant patients, it must be a very stable cell population. However, as studies have shown that Tregs may lose their suppressive ability as well as potentially converting to a Th17-like cell whereby they produce high amounts of IL-17 to promote a pro-inflammatory microenvironment (H. J. P. M. Koenen et al. 2008; X. O. Yang et al. 2008) suggests that Tregs may change once inside a transplant patient. Thus, a combinatory or a more robust therapy along with Tregs may be required to ensure an immunoregulatory environment.

1.7 Extracellular Vesicles

The paradigm of intercellular communication which is a vital feature of multicellular organisms has until recently been anticipated to be mediated via direct cell-to-cell contact or via the exchange of secreted molecules. An increasing number of experimental reports have revealed a third mechanism of intercellular communication via the cell-to-cell transfer of EVs. EV is a collective umbrella term used to describe various types of small particle structures secreted from cells into the extracellular space.

The nomenclature used in early EVs research has been lax and many earlier published reports use EV terminology interchangeably, although no consensus was set to define categories of EVs. However, it is now becoming clearer and more appreciable to classify EVs into 3 main categories; exosomes, microvesicles and apoptotic bodies.

1.7.1 Brief history of EVs

Originally in 1981, the term ‘exosomes’ was used to describe vesicles ranging from 40-1000nm that are released from cultured neoplastic cells (Trams et al. 1981), although their subcellular location of release was unclear.

Then in 1983, Harding *et al.* (Harding, Heuser, and Stahl 1983) and Pan and Johnstone (Pan and Johnstone 1983) published two independent papers describing the release of 40-100nm-sized EVs that were released during reticulocyte differentiation as a result of

multivesicular endosome (MVE) fusion with the plasma membrane, although in these publications the term ’exosomes’ was not used but ‘vesicles’. Later in 1989, Johnstone *et al.* coined these late-endosome-derived vesicles ‘exosomes’ (Johnstone, Bianchini, and Teng 1989). However, after this publication by Johnstone *et al.* in 1989, the interest in exosomes research declined due to the scepticism surrounding the notion that late endosome contents were considered to be destined for degradation by the lysosome and thus held doubtfulness in functional roles (Johnstone, Bianchini, and Teng 1989).

The seminal study by Raposo *et al.* in 1996 re-ignited interest in exosomes research when the authors discovered that human and mouse B cells secrete antigen presenting vesicles which induced antigen-specific MHC class II-restricted T cell responses and suggested their potential functional *in vivo* role (G Raposo et al. 1996). Since Raposo *et al.*’s publication (G Raposo et al. 1996), many groups have reported EVs release from various cell types including immune and non-immune cells.

1.7.2 Categories of EVs

As abovementioned, EVs classification is generally divided into 3 main categories of exosomes, microvesicles and apoptotic bodies which are defined by their size and biogenesis pathway. Exosomes are 30-100nm sized in diameter with a reported maximum size of 150nm (Colombo, Raposo, and Théry 2014) and formed within endocytic cellular compartments called multivesicular bodies (MVB), whereby the MVB fuses with the plasma membrane allowing their release (Clotilde Théry, Zitvogel, and Amigorena 2002); microvesicles are 500-1000nm sized in diameter and derived from the outward budding of the plasma membrane (Willms et al. 2016), although smaller vesicles approximately 100nm sized can also bud from the plasma membrane (A. M. Booth et al. 2006) and apoptotic bodies are 50-2000nm sized and derived from the outward blebbing and disintegration of apoptotic cells (Willms et al. 2016).

Notably, other types of EVs have been reported in literature such as ectosomes (Hess et al. 1999), shedding vesicles (Cocucci, Racchetti, and Meldolesi 2009), microparticles (György et al. 2011) and exosome-like vesicles (Islam et al. 2008). But it is unclear whether these EVs fall into one of the 3 main categories described above, or whether these are another category or subtype of a category of EVs. Thus, there is a current

request in the EVs research field to standardise the terminology and methodology used to isolate EVs and criteria followed in order to accurately define each category of EVs.

Most cell types release EVs (Clotilde Théry, Zitvogel, and Amigorena 2002). EVs can be found in various bodily fluids such as urine, blood, plasma, serum, semen, breast milk, saliva, amniotic fluid, cerebrospinal fluid, ascite fluid and bile (Clotilde Théry, Zitvogel, and Amigorena 2002; Colombo, Raposo, and Théry 2014; G Raposo et al. 1996).

1.7.3 EVs biogenesis

The endocytic pathway is a highly regulated process. The initial step of exosomes biogenesis involves the inward budding of the plasma membrane to form an early endosome which is aided by a variety of protein complexes such as the endosomal sorting complexes required for transport (ESCRT). In close proximity to these early endosomes, an assembly of extracellular receptors, CD markers, cytosolic components or other cellular components are recruited to these areas. The early endosome then invaginates inwards to start the formation of intraluminal vesicles in their lumen. Whilst the intraluminal vesicles are being formed the encapsulation of specific cellular components are sorted and packaged into the intraluminal vesicles. Once all the intraluminal vesicles are formed, the endosome is now known as MVB. The MVB migrates to the plasma membrane whereby it fuses with the plasma membrane to release the intraluminal vesicles into the extracellular space. Once the intraluminal vesicles are released from the cell they are known as exosomes (Figure 1.3). Alternatively, the MVB are destined to fuse with lysosomes for their degradation.

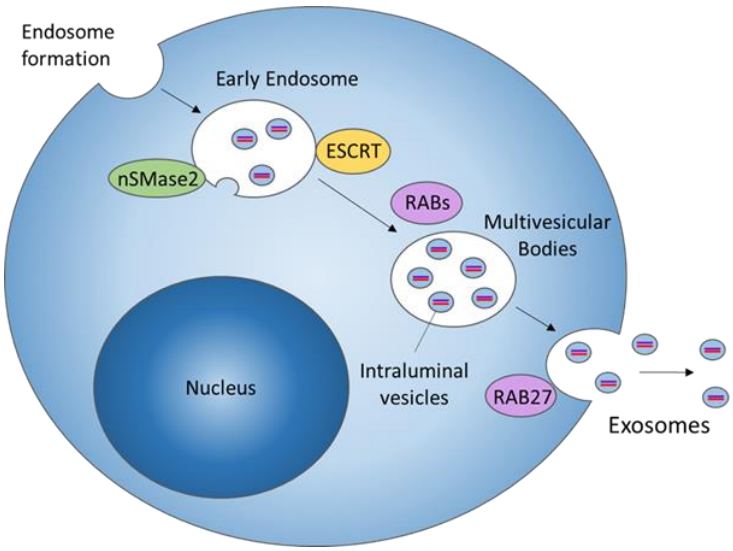


Figure 1.3- Biogenesis of exosomes

Schematic diagram illustrating the biogenesis of exosomes and the involved proteins and molecules during this exosome secretion process.

The biogenesis of microvesicles is different to that of exosomes. Microvesicles biogenesis involves the assembly of molecular cargo to the limiting plasma membrane. The rearrangement of membrane lipids and the interaction of contractile machinery at the plasma membrane level permits the vesicle outward budding process (D'Souza-Schorey and Clancy 2012). During the microvesicles formation process, specific biological components are selected to enter the microvesicles and once formed, the microvesicles are blebbed or pinched from the plasma membrane to allow their release into the extracellular space.

The biogenesis of apoptotic bodies only occurs during programmed cell death (Akers et al. 2013). The cells that are programmed to die will go through various stages which start with the condensation of the nuclear chromatin, subsequently the membrane starts to disintegrate by blebbing, and during the blebbing process cellular components are packaged. These membrane blebs derived from dying cells are known as apoptotic bodies.

1.7.4 EVs characteristics and content

Due to some overlap in exosomes, microvesicles and apoptotic body sizes and protein content, it makes the discrimination of these different types of EVs challenging.

Exosomes are saucer shaped vesicles with a lipid bilayer and float at a density of 1.13-1.19g/ml^{-1} in sucrose gradients (Clotilde Théry, Zitvogel, and Amigorena 2002). Thery *et al.* explains that exosomes comprise of proteins derived from the cell cytosol, plasma membrane and endosomal compartments but not from endoplasmic reticulum, Golgi apparatus, mitochondria or the nucleus (Clotilde Théry, Zitvogel, and Amigorena 2002). However, in contrast, Pols and Klumperman state that CD63 tetraspanin is synthesised in the endoplasmic reticulum (Pols and Klumperman 2009), and CD63 is a common marker of exosomes (Pols and Klumperman 2009; Clotilde Théry, Zitvogel, and Amigorena 2002). Furthermore, mitochondrial-derived deoxyribonucleic acid (DNA) can be found in exosomes (Sansone et al. 2017). This discrepancy could be due to different cell sample type and highlights that EVs may vary in content depending on the parent cell type.

On the contrary, exosomes indeed contain various proteins that are common irrespective of what cell type they originate from. For example, mouse and human DC-derived exosomes contain protein of which 80% of these are conserved between the two species (Clotilde Théry, Zitvogel, and Amigorena 2002). However, they also can carry proteins that reflect the specific parent cell (Ventimiglia and Alonso 2016). In particular, the contents that get packaged into exosomes depend on the parent cell lineage and state (whether it is resting or activated, immature or mature) and /or the stimulus type (Robbins and Morelli 2014).

Noteworthy, cells can host remarkably different populations of MVBs. For example, in epithelial cells, the EVs that were released from the apical and basolateral sides of the cells differed in their contents even though these EVs are derived from the same cell (Qiuhong Chen et al. 2016). Indeed, it is widely reported that exosomes are heterogenous and their composition is influenced by modifications in culture conditions and differentiation states of the secreting cells (Colombo, Raposo, and Théry 2014). For instance, the presence of inflammatory signals such as lipopolysaccharide (LPS), TNFα

and IFNγ can affect the protein and ribonucleic acid (RNA) that get packaged into EVs released by DCs (Segura et al. 2005).

EVs can encompass various biological materials including micro-RNA (miRNA), mRNA, proteins, lipids, cytokines, MHC molecules, CD molecules, adhesion molecules, heat shock proteins, signal transduction proteins, trafficking proteins, antigens/peptides, enzymes, chemokines and other biological materials. Due to the proficiency in carrying biological material and their mobility both locally and systemically it marks EVs as efficient transport vehicles that conduct important cell-to-cell communication.

EVs encompass various proteins consisting of CD63, CD81, CD9, ESCRT, neutral sphingomyelin phosphodiesterase (nSMase), Rab proteins, heat shock proteins, lipid and nucleic acid amongst other biological materials. There are various ways to isolate and describe EVs, however the minimal requirements of biochemical, biophysical, phenotypical and functional criteria including the methodical approach to EV isolation and characterisation are discussed in detail by Lotvall *et al*. (Lötvall et al. 2014) and Witwer *et al*. (Kenneth W. Witwer et al. 2013).

1.7.4.1 Expression of CD63, CD81 and CD9 in EVs

Of the most commonly found proteins in EVs are CD63, CD81 and CD9. These are part of the transmembrane 4 superfamily, which is also known as the tetraspanin family. These are cell surface proteins that have 4 hydrophobic domains and are mediators of various signal transduction processes.

These tetraspanins interact with MHC molecules and integrins which aid their role in organising large molecular complexes and membrane components to facilitate the assembly of signalling molecules required for essential cellular events (Clotilde Théry, Zitvogel, and Amigorena 2002). These cellular events include the regulation of cell development, activation, growth and proliferation.

CD63 is the first characterised tetraspanin (Pols and Klumperman 2009). The enrichment of tetrespanins in exosomes is from 7-124 folds higher compared to their parent cells (Andreu and Yáñez-Mó 2014). CD63 and CD81 are the most commonly used marker of exosomes. Although most reports describe CD63 as exosome-specific, it was

recently discovered that CD63 and CD81 can be present in exosomes, microvesicles and apoptotic bodies derived from the same parental cells (Crescitelli et al. 2013), rendering CD63 and CD81 not exosome-specific per se. The presence of CD9 in EVs are not consistently found in exosomes (Crescitelli et al. 2013). On the other hand, primary B cell-derived exosomes contain CD9 and CD81 but did not express CD63 (Saunderson et al. 2008), thus highlighting the heterogeneity of EV contents.

The function of these tetraspanins is most well-documented in terms of their relation to the parent cells as opposed to their function in the EVs. Nonetheless, these tetraspanins has been described to be involved in EVs cargo sorting (including proteins and RNA), EVs targeting and uptake and antigen presentation (Andreu and Yáñez-Mó 2014). Tetraspanins organised in tetraspanin enriched microdomains (TEMs) on immune cell's surface are involved in antigen presentation by recruiting proteins to TEMs forming complexes to initiate immune synapse formation. The major role CD81 plays in this has been highlighted by Mittelbrunn *et al.*, where the authors describe that CD81 is found in the central supramolecular activation complex (c-SMAC) (Mittelbrunn et al. 2002), and facilitates the process of immune synapse formation in the T cell via the interaction of CD3 and intercellular adhesion molecule 1 (ICAM-1) (Rocha-Perugini et al. 2013), thus facilitating an efficient T cell activation. In APCs, CD81 has been suggested to interact with MHC molecules (Wright, Moseley, and van Spriel 2004), which can enhance formation of MHC-II multimer and enhance antigen presentation (Unternaehrer et al. 2007; Poloso, Denzin, and Roche 2006; Kropshofer et al. 2002).

Edgar *et al.* showed that CD63 was essential in the biogenesis of small intraluminal vesicles in MVB of HeLa cells (Edgar, Eden, and Futter 2014), thus CD63 is not only a marker of EVs but may also play an essential role in their biogenesis.

1.7.4.2 ESCRT and nSMase

The ESCRT consists of approximately 20 proteins assembled into 4 complexes (ESCRT-0, -I, -II and -III) which is associated with accessory proteins such as Alix and tumour susceptibility gene 101 (Tsg101).

The ESCRT-0 complex identifies and sequesters ubiquitinated transmembrane proteins in the endosomal membrane, whilst ESCRT-I and -II complexes are involved in the

membrane biogenesis of buds with selected cargo. The ESCRT-III complex then promotes the vesicle scission (Hanson and Cashikar 2012). Alix is involved in exosome biogenesis and sorting of syndecans via its interaction with syntenin (Baietti et al. 2012). Babst *et al.* demonstrated that Tsg101 mutant cells were defective in the transport of membrane proteins to the late endosomal compartments (Babst et al. 2000), which highlights the important role Tsg101 plays in the normal trafficking of membrane proteins in the endocytic pathway.

However, in the cells that lack ESCRT machinery, MVBs and exosomes are still able to be formed and $CD63^+$ EVs produced, suggesting an exosome biogenesis pathway that is ESCRT-independent (Stuffers et al. 2009), and as explained above could be CD63-driven.

This ESCRT-independent pathway involves nSMase. In the nSMase pathway, nSMase hydrolyses sphingomyelin into ceramide, and phospholipase D2 hydrolyses phosphatidylcholine into phosphatidic acid. These two lipid metabolising enzymes can produce lipids in the limiting membrane of MVB which initiates the inward invagination of intraluminal vesicles which thus are independent of the ESCRT pathway. Whether the ESCRT or nSMase pathway of exosome biogenesis occurs can influence what cargo gets packaged into the contents of exosomes (Colombo, Raposo, and Théry 2014).

1.7.4.3 Rab proteins

Rab proteins are essential for transport of endosomal structures in cells. Rab proteins play a role in vesicle budding, vesicle movement with actin and tubulin networks and membrane fusion of MVBs. There are approximately 70 different Rab proteins in humans, which most are involved in vesicles trafficking. In 2002, Savina *et al*. published the first report of Rab protein Rab11 to be involved in exosome secretion, the authors showed that Rab11-deficient K562 cells had a significant decrease of exosomes production (Savina, Vidal, and Colombo 2002). Later studies showed that Rab35 (C. Hsu et al. 2010) and Rab27αβ (Ostrowski et al. 2010) were also involved in the fusion of late endocytic compartments and MVBs with the plasma membrane to initiate secretion of intraluminal vesicles as exosomes.

1.7.4.4 Heat shock proteins

Heat shock protein (HSP)70 and HSP90 are commonly found in EVs secreted from various cell types. These ubiquitous proteins play a role in antigen presentation by binding to antigenic peptide and loading them onto MHC molecules (Srivastava 2002).

1.7.4.5 Lipids

EVs contain a mixture of lipids, mainly consisting of ceramide, sphingomyelin, cholesterol, phosphatidylserine and saturated fatty acids. These components together with flotillins provide EVs with a detergent-resistance property (Wubbolts et al. 2003; Colombo, Raposo, and Théry 2014) and thus this property adds further protection from degradation whilst in transit to their destination target cell.

1.7.4.6 Nucleic acids

Almost all cells harbour miRNAs, but the miRNA repertoire of different cell types vary tremendously (Turner, Schnorfeil, and Brocker 2011; Ha 2011). Previously, it was thought that mainly cells carried nucleic acids until in 2007, Valadi *et al*., who were the first group to demonstrate that exosomes also carry nucleic acids (Valadi et al. 2007). Namely these were mRNAs and miRNAs and these exosomal-associated RNA molecules served as a novel means of cell-to-cell communication. This breakthrough study showed that human cells which were treated with mouse exosomes, contained mouse proteins. The presence of mouse protein in the human cells could only have derived from the mouse exosomes. The mouse exosomes contained the relevant mRNA coding for this protein which indicated that mRNA transported in exosomes were functional and had been translated into protein in the target cell (Valadi et al. 2007).

Noteworthy, miRNA can be associated to Ago2 protein (Arroyo et al. 2011) and high-density lipoproteins (Vickers et al. 2011) which these proteins aid their transport in a cell-free and EV-free manner.

Exosomal RNA consists mainly of small RNA including mRNA and miRNA but lacks 18S and 28S RNA which are found in cellular RNA (Crescitelli et al. 2013). Nonetheless, small

ribosomal RNA, specific transfer RNA (tRNA) fragments, vault-RNA and Y-RNA have also been found within EVs (Colombo, Raposo, and Théry 2014).

miRNA is a non-coding RNA molecule that is approxiamately 22 nucleotides long and function in RNA silencing and post-transcriptional regulation of gene expression by inhibitng mRNA translation into protein. Several studies have demostrated that EVs are a rich source of miRNAs, more often enriched compared to their parent cell miRNA contents (Clotilde Théry, Zitvogel, and Amigorena 2002; Robbins and Morelli 2014; Villarroya-Beltri, Gutiérrez-Vázquez, Sánchez-Madrid, et al. 2013).

Importantly, EVs offer a protective route of miRNA transfer between cells. As EVs have a lipid bilayer of ceramide and cholesterol and can encapsulate contents and evade degradation by the complement system by having the presence of CD55 and CD59 on its surfaces together can evade degradation by proteinases and ribonucleases (RNase) (Robbins and Morelli 2014).

The miRNA cargo of EVs is not a random process but a regulated sorting mechanism is in place to select specific miRNAs to be packaged into EVs. This was demonstrated by Villarroya-Beltri *et al*. in 2013. These authors showed that sumoylated hnRNPA2B1 controls which miRNAs are packaged into exosomes through its interaction with specific motifs (Villarroya-Beltri, Gutiérrez-Vázquez, Sánchez-Cabo, et al. 2013).

Importantly, EVs are not mere smaller versions of the parent cells (Srivastava 2002). Blanchard *et al.* demonstrated the T cell derived exosomes lacked the abundant parent cell-surface CD45 and CD28 but did however contain TCR/CD3ζ complexes. Given that the parent T cell has high abundance of CD45, CD28 and TCR/CD3ζ complexes, but only TCR/CD3ζ complexes were found on the exosomes suggests a specific packaging mechanism in the cargo of EVs (Blanchard et al. 2002). Indeed, this is also the case for other cell types such as DCs. Thery *et al.* demonstrated that DCs which are rich in Fc receptors produced exosomes which lacked these cell surface molecules (C Théry et al. 2001), again suggesting a regulated packaging mechanism of EVs.

1.7.5 EVs interaction with target cells

The binding of EVs to the target cell surface can be facilitated by the presence specific receptors. For example, Nolte-'t *et al.* demonstrated that activated T cells bind DC EVs through the interaction of leukocyte function-associated antigen-1 (LFA-1) rather than on TCR specificity (Nolte-'t Hoen et al. 2009). The modest binding of EVs to target cells may be adequate to initiate biochemical changes in the target cell, as demonstrated by Denzer *et al*. (Denzer et al. 2000) and Segura *et al*. (Segura et al. 2007), whereby APC EVs which displayed MHC-peptide complexes can activate antigen specific T cells. In other scenarios, the contents of EVs must be transported inside the target cells. The internalisation of EVs into target cells can occur through various phagocytosis and endocytosis mechanisms. The internalisation of EVs is necessary for its contents such as miRNA to be released into the target cell gene processing machinery for gene silencing to occur (Colombo, Raposo, and Théry 2014).

The delivery route of EVs towards target cells may be protected not just by the lipid bilayer that surrounds them but also by functional markers. For example, Clayton *et al.* demonstrated that APC exosomes expression of CD55 and CD59 protected the exosomes from complement-mediated degradation (Aled Clayton et al. 2003).

1.7.6 Immune cell derived EVs

The secretion of EVs from T cells and Tregs are in a calcium (Ca^{2+})-dependent manner (Okoye et al. 2014; Blott and Griffiths 2002), and thus cell activation releases their EVs. On the contrary, other cell types such as Epstein-Barr virus (EBV)-transformed B cells, DCs and epithelial cells constitutively release EVs (G van Niel et al. 2001; C Théry et al. 1999; G Raposo et al. 1996).

Several studies have shown that immune and non-immune cells release EVs that can modulate the immune system, mediating an activated or a suppressed immune response (Robbins and Morelli 2014), thus highlighting the potential of EVs treatment in immune therapy. EVs can act as both anti-inflammatory and pro-inflammatory factors depending on the cell from which they originate from and from the parent cell status (resting/activated or immature/mature) and the stimulus used to release them (if any).

1.7.7 T cell derived EVs

The 7 publications described in section 1.7.8 are the only publications to date and to the best of my knowledge, demonstrating Treg EVs. However, a multitude of research has been conducted on T cell EVs and contrary to Treg EVs which all the publications so far have shown they are immunosuppressive; T cell EVs can both suppress and promote the immune response. The pleiotropic role of T cell EVs could be due to various factors including their parent cell status, packaged contents and their target cell.

T cell EV contents have been explored more extensively than Treg EV contents. In 2002, Blanchard *et al.* demonstrated that T cell derived exosomes comprise the TCR/CD3ζ complex which give T cell exosomes the potential to deliver activation signals to cells bearing the complementary peptide/MHC complexes. These exosomes also contained proteins of the transduction pathway, Src-like tyrosine kinases and adhesion molecules (Blanchard et al. 2002). Whether these proteins are functional in these exosomes was not clear at the time. Since this publication in 2002, more studies have unravelled the roles and functions of T cell EVs.

1.7.7.1 Immunosuppressive T cell EVs

In 2004, Nolte-'t Hoen *et al.* demonstrated that MHC-II/peptide complexes-bearing vesicles derived from anergic T cells can be taken up by APCs and endowed them with immune-suppressive properties. These vesicles also contained CD80, CD86, CD2, CD54 and CD25 which may have contributed to their immunosuppressive ability. Furthermore, the transfer of bioactive material from T cells to APCs does not require antigen-specific cell-to-cell contact but can be mediated via vesicles suggested that vesicles can play an important role in immune responses (Nolte-'t Hoen et al. 2004).

In 2011, Zhang *et al.* demonstrated that ovalbumin (OVA)-antigen-specific $CD4^+$ T cell derived exosomes can inhibit DC_{OVA} mediated $CD4^+$ T cell proliferation *in vitro* and functioned *in vivo* to inhibit $CD8^+$ cytotoxic T lymphocyte (CTL) responses. These T cell exosomes expressed CD4, TCR, LFA-1, CD25 and FasL (H. Zhang et al. 2011), whether these molecules contributed to the immunosuppressive responses was unclear. Nonetheless, these authors demonstrated that the interactions of the peptide/MHC II/TCR and CD54/LFA-1 of DC_{OVA} and $CD4^+$ T cell exosomes facilitates the uptake

exosomes into these DCs. Thus, demonstrating that exosomes express markers that can enable them to enter target cells to modulate their function (H. Zhang et al. 2011).

In 2011, Mittlebrunn *et al.* demonstrated that T cells release miRNA-containing exosomes into APCs in a unidirectional manner that is antigen-driven and occurs during the immunological synapse. APCs which received these exosomal miRNA had their gene expression modulated (Mittelbrunn et al. 2011), supporting the notion that exosomes safely deliver biological materials which are functional in the target cells. Specifically, miR-355 was shown to be transferred from T cells to their exosomes and this exosomal miR-355 was delivered into APCs which downregulated SOX-4 three prime untranslated region (3'UTR) mRNA expression levels, a target of miR-355. Hence, these authors experimentally demonstrated that exosomes carried and offered a protected vehicle of miRNA passage into target cells.

In 2013, Bryniarski *et al.* demonstrated that $CD8^+$ suppressor T cell exosome-like nanovesicles can be manipulated by coating the surface of exosomes with antibody light chains to facilitate their uptake via an antigen specific manner into target Teffs. Furthermore, these nanovesicles can be transfected with specific miRNA. Additionally, these authors discovered that nanovesicle-associated miR-150 as a mediator of immunosuppression. These nanovesicles carried and delivered miR-150 into antigen specific targeted cells and was shown to inhibit allergic contact dermatitis in mouse models (Bryniarski et al. 2013) and thus highlights that manipulation of nanovesicles is possible and importantly that these manipulated nanovesicles can function *in vivo.*

Not only can T cell EVs modulate target cell functions by inhibiting their cellular machinery but can also cause target cell death as a means of suppressing their cell expansion numbers. For instance, various studies have shown that T cells can release APO2L (Martínez-Lorenzo et al. 1999; Monleón et al. 2001) and membrane-associated and FasL-containing exosomes which can induce target cell death (Monleón et al. 2001; Alonso et al. 2005; Martínez-Lorenzo et al. 1999).

1.7.7.2 Immune promoting T cell EVs

However, as explained above, T cell EVs can also promote the immune response.

In 2012, Wahlgren *et al*. showed that activated $CD3^+$ T cells released exosomes which work in unison with IL-2 to promote proliferation of resting autologous T cells and modulate their cytokine production profiles (Wahlgren et al. 2012). Interestingly, exosomes together with IL-2 generated secretion of more cytokines and at higher concentrations than exosomes or IL-2 alone. Noteworthy, chemokine ligand (CCL)-4 was secreted by T cells stimulated with exosomes plus IL-2 together but CCL4 was not secreted if the treatment consisted of exosomes or IL-2 alone.

Various studies have shown that T cell EVs can promote inflammation by inducing monocytes to produce pro-inflammatory cytokines TNFα and IL-1β (Scanu et al. 2008; Carpintero et al. 2010; Zakharova, Svetlova, and Fomina 2007).

Taken together, various studies have shown that immune cell-derived EVs modulate the immune response by altering the cytokine production of target cells to provide either an anti-inflammatory or pro-inflammatory micro-environment.

In recent years, Tregs have been shown by us and other groups to release EVs.

1.7.8 Treg cell derived EVs

1.7.8.1 Mouse

Importantly, to date, various groups have demonstrated that mouse (Lesley Ann Smyth et al. 2013; Tung et al. 2018; Okoye et al. 2014), rat (X. Yu et al. 2013; Aiello et al. 2017) and human (Torri et al. 2017; Azimi et al. 2018) Tregs release EVs that are immunomodulatory. The host laboratory, in 2013 were the first to demonstrate that mouse Tregs release immunomodulatory exosomes upon TCR activation (Lesley Ann Smyth et al. 2013). These Treg exosomes were able to suppress the proliferation of $CD4^+CD25^-$ T cells *in vitro* and significantly reduced their production levels of pro-inflammatory cytokines IL-2 and IFNγ, thus creating an anti-inflammatory microenvironment. The presence of the ectoenzyme CD73 on Treg exosomes was discovered to contribute in part to their immunosuppressive ability (Lesley Ann Smyth et al. 2013), although other molecules may also be involved in their

immunosuppression. These authors explained that CD39 is a cell surface located enzyme found on Tregs that catalyses pro-inflammatory ATP into AMP, which CD73, found on Treg exosomes converts to anti-inflammatory adenosine. Activated Teffs express adenosine receptors $A_{2a}R$ (Chhabra et al. 2012; L. Han et al. 2017; Leone et al. 2018) which facilitates the uptake of this exosome-converted adenosine and subsequently this initiates intracellular cAMP production resulting in decreased cytokine production and thus preventing T cell pro-inflammatory responses (Baietti et al. 2012).

CD73 expression on mouse Treg exosomes contributed to their immunosuppressive ability (Lesley Ann Smyth et al. 2013), but also other molecules are likely involved. This was addressed in 2014 by Okoye *et al.* Firstly, the authors confirmed the findings of the host laboratory that mouse $CD4^+$ $CD25^+$ Tregs released exosomes upon their activation (Okoye et al. 2014). Secondly, Okoye *et al.* also suggested that activated Treg cells released more $CD63^+$ exosomes per cell compared to activated or stimulated naïve $CD4^+$ cells; naïve $CD8^+$ cells; B cells; Th17 cells or Th1 cells, and their release are regulated by IL-2, amphiregulin and ATRA (Okoye et al. 2014). On deeper analysis, the authors found that Treg cell release of exosomes were influenced by changes in intracellular calcium, hypoxic conditions and the biosynthesis of ceramide (Okoye et al. 2014), similar to the findings by other groups showing that these factors also regulate exosome release (Savina et al. 2003; H. W. King, Michael, and Gleadle 2012; Trajkovic et al. 2008).

The mouse Treg exosomes contained pre-mature miRNA, mature miRNA and mRNA, of which the Treg exosomal miRNA repertoire were differential compared to the miRNA profile found in the parent cells, Th1 cell exosomes or Th2 cell exosomes (Okoye et al. 2014), suggesting potential diverse roles for Treg exosomes. Indeed, mouse Treg exosomes transferred Let-7d from the parent Treg cell into Th1 cells and inhibited target mRNA resulting in their suppressed proliferation *in vitro* and importantly, these EVs functioned *in vivo* to prevent autoimmune disease.

Recently, another mouse Treg EV paper was published by myself and the host laboratory in 2018 (Tung et al. 2018). We demonstrated the novel finding that mouse Treg EVs are acquired by BM-DCs and altered their cytokine production. Namely, these Treg EV-treated bone marrow-derived DCs (BM-DCs) significantly reduced the production of pro-inflammatory IL-6 whilst increasing their immunosuppressive IL-10 production levels upon LPS treatment (Tung et al. 2018). These mouse Treg EVs

contained miR-150-5p and miR-142-3p, which was transferred into BM-DCs. Given that both miRNAs have previously been reported to affect DC cytokine production (Lesley A Smyth et al. 2015), it was possible that these EV-associated miRNAs that were transferred into BM-DCs modulated their function by affecting their cytokine production, thus creating a 'tolerogenic' phenotype. These findings will be presented and discussed in Chapter 3.

1.7.8.2 Rat

Rat Tregs releasing immunomodulatory EVs have also been reported in literature (X. Yu et al. 2013; Aiello et al. 2017).

In 2013, Yu *et al.* demonstrated that the adoptive transfer of rat Treg derived exosomes into a rat model of kidney transplantation prolonged the survival of the allograft (X. Yu et al. 2013). In particular, donor Treg derived exosomes were more suppressive towards T cell proliferation and prolonged allograft survival longer than autologous Treg derived exosomes. Conversely, these authors also showed that B cell derived exosomes induced rather than suppressed T cell proliferation, possibly due to the presence of allergen-derived peptides that induce T cell responses (X. Yu et al. 2013). However, the molecular mechanism driving rat Treg exosomes suppressive ability was not addressed by these authors.

Nonetheless, in 2017, Aiello *et al*. provided deeper insights into the role of rat Treg derived EVs (Aiello et al. 2017). The authors used rat DCs that maintained their immature state by genetic transfer of dominant negative form of inhibitor of nuclear factor kappa-B kinase subunit beta (IKK2) and co-cultured these with T cells. This co-culture produced a unique population of Tregs (dnIKK2-Treg). These Tregs produced immunomodulatory EVs that suppressed T cell proliferation. These EVs contained miRNA including miR-503 which they suggest when transferred into target cells contributed to blocking cell cycle progression, additionally, nitric oxide synthase (iNOS) enzyme was found inside Treg EVs which can induce apoptosis in target cells. Furthermore, these Treg EVs converted naïve T cells into induced Tregs, demonstrating the potency of EVs in modulating target cell biology (Aiello et al. 2017). Importantly,

these rat Treg EVs, similar to the finding of Yu *et al*. (X. Yu et al. 2013), prolonged kidney allograft survival.

1.7.8.3 Human

During the course of my PhD studies, it was published that human Tregs, akin to their murine counterparts, also release immunomodulatory EVs (Torri et al. 2017; Azimi et al. 2018).

In 2017, Torri *et al.* were the first group to report that human Tregs release immunomodulatory EVs (Torri et al. 2017). These authors isolated EVs from Th1, Th17 and Treg cells and compared their miRNA repertoire. miR-146a-5p, miR-150-5p and miR-21-5p were identified as being enriched in Treg EVs. The authors described that Treg EVs suppressed T cell proliferation, which they linked to Treg EV-associated miR-146a-5p given that two relevant targets of miR-146a-5p, Stat1 and Irak2 expression was significantly decreased in target cells that were treated with Treg EVs compared to Th17 EVs (which contained significantly less miR-146a-5p expression levels). Stat1 and Irak2 are two genes that are up-regulated upon stimulation of naïve T cells (Torri et al. 2017), and thus the suppressed expression of these genes may prevent their maturation.

In 2018, Azimi *et al.* (Azimi et al. 2018) confirmed the findings of Torri *et al*. (Torri et al. 2017) that human Treg exosomes are immunomodulatory. Azimi *et al.* compared the Treg exosomes derived from healthy controls and MS patients and found that both types of exosomes were suppressive and induced apoptosis of conventional T cells, but the MS Treg exosomes were less potent than that of healthy controls (Azimi et al. 2018), suggesting that they were impaired.

1.7.9 EVs that induce Tregs

EVs derived from other cells types can induce Treg cell populations. For example, the study by Cai *et al.* demonstrated that TGF-β1 gene-modified DCs exosomes can induce $CD4^+$ $FoxP3^+$ Tregs via TGF-β1 *in vitro* and *in vivo.* These authors found increased iTregs in mesenteric lymph nodes of the inflammatory site of inflammatory bowel disease

(IBD) mice. These iTregs prevented Th17-mediated inflammatory damage in IBD murine models better than TGF-β1 cytokine alone (Z. Cai et al. 2012).

In 2008, Wang *et al.* reported that thymus-derived exosome-like particles also induce FoxP3^{+} Treg cell populations, which was likely driven by the presence of TGF-β in these particles. These particles induced CD4^{+} CD25^{-} T cells to convert into functionally suppressive Tregs capable of suppressing the proliferation of CD4^{+} CD25^{-} T cells both *in vitro* and *in vivo* (G.-J. Wang et al. 2008).

Furthermore, EVs derived from other cell types such as mesenchymal stem cells (MSCs) stimulated with TGF-β and IFNγ promoted Treg differentiation (Q. Zhang et al. 2018) and EVs derived from tumour cells can also induce, expand and enhances Treg population and their suppressive functions (Szajnik et al. 2010).

1.7.10 EVs in transplantation

EVs have been involved in various aspects of transplantation, for example as a means of cell-free therapy. As previously mentioned, the adoptive transfer of rat Treg EVs can prolong rat kidney transplant survival (X. Yu et al. 2013; Aiello et al. 2017). Whether human Treg EVs can also similarly prolong allograft survival has thus far not been demonstrated and is one of the aims of my PhD thesis. Nonetheless, EVs derived from other cell types have shown potential in promoting allograft survival. For example, Li *et al*. showed that immature DC exosomes given together with rapamycin induce transplant tolerance in mouse heart allografts. Donor DC exosomes prolonged the allograft survival longer than recipient DC exosomes. Furthermore, the authors showed that the activation and proliferation of alloreactive T cells were suppressed more potently when donor DC exosomes were used in combination with rapamycin compared to donor DC exosomes treatment alone. The combined treatment induced CD4^{+}CD25^{+} Treg cell populations in the spleen which may explain, in part, the inhibition of alloreactive T cells (X. Li et al. 2012). In 2016, Song *et al*. (Song et al. 2016) reported a similar finding to Wang *et al*. (G.-J. Wang et al. 2008) that exosomes can covert Teffs to Tregs. Song *et al*. demonstrated that donor exosomes can induce Tregs *in vivo* which were donor antigen specific and capable of inhibiting immune inflammation in the allograft heart. These authors isolated peripheral exosomes from the mouse serum and

identified the presence of matrix metallopeptidase 1a (MMP1a), which this induced $CD4^+$ T cell to express FoxP3 and TGF-β via inhibiting the Th2 transcription factor GATA3. Hence, this study showed that exosomes can polarise $CD4^+$ T cells and convert Th2 cells to Tregs (Song et al. 2016).

In rat models of cardiac allograft transplantation, the adoptive transfer of donor DC exosomes together with short-term suboptimal doses of immunosuppression that blocks DC maturation can induce donor-specific allograft tolerance by inhibiting the anti-donor proliferative responses (Peche et al. 2006).

However, on the other end, EVs can induce allo-immune T cell responses that exacerbate transplantation outcomes. For instance, the passenger leukocyte theory described in 1957 by Snell (Snell 1957), has recently been revisited by Marino *et al*. (Marino et al. 2016) and Liu *et al*. (Q. Liu et al. 2016). Marino *et al*. demonstrated that donor exosomes which carry donor MHC are taken up by recipient APCs (semi direct-pathway of allorecognition) and these cross-dressed recipient APCs could induce pro-inflammatory alloimmune T cell responses in transplant models, thus APCs cross-dressed with exosomes could trigger the transplant rejection process (Marino et al. 2016). Liu *et al*. also showed a similar finding in cardiac transplants in mice (Q. Liu et al. 2016).

1.8 Hypothesis and Aims

1.8.1 Hypothesis

Mouse Treg EVs can transfer miRNAs into target bone marrow-derived DCs (BM-DCs) to modulate their phenotype and cytokine production to promote an immunoregulatory microenvironment.

Human Tregs release EVs that contain miRNAs, these miRNAs are transferred into target T cells (T responders (Tresps)). Human Treg EVs can suppress the proliferation of Tresps and modulate their production of pro-inflammatory cytokines. These human Treg EVs can also function *in vivo* to promote transplant allograft tolerance.

1.8.2 Aims

This PhD project aimed to further characterise mouse and human Treg derived EVs. The following aims were set:

(1) Investigate the miRNA content of mouse Treg EVs and assess if they can be transferred into BM-DCs and alter BM-DC cytokine production profiles.
(2) Characterise human Treg EVs and test their *in vitro* suppressive ability and their ability to modulate the cytokine production profile by Tresps.
(3) Investigate the miRNA repertoires contained within human Treg EVs and perform bioinformatics to predict which mRNAs these miRNAs can target.
(4) Assess the efficacy with which human Treg EVs protect against alloimmune-mediated damage in human skin allografts *in vivo.*

Chapter 2
Materials and Methods

Chapter 2 - Materials and Methods

2.1 Mice subjects

2.1.1 Subjects

Female C57BL/6 (B6) and BALB/c mice (approximately 8 weeks old) were purchased from Harlan, UK.

BALB/c RAG2$^{-/-}$ γc$^{-/-}$ (BRG) mice (approximately 10-12 weeks old), knockout for the recombinant-activating gene 2 (RAG-2) and the IL-2 common gamma chain (γc) genes were bred in house in Biological Sciences Unit of New Hunt's House, King's College London, UK.

B6, BALB/c and BRG mice were all housed in the Biological Sciences Unit and kept under specific pathogen free conditions. All animal experimental procedures were conducted under sterile conditions in accordance to Home Office regulations under the project licence (PPL) number 70/7302.

Rab27α$^{ashen/ashen}$ β$^{-/-}$ knock-out (Rab27DKO) mice, knockout for the Rab27 member (consisting of two closely related homologs of Rab27α and Rab27β) of the Rab subfamily of GTPases were generated in a B6 background (Tolmachova et al. 2007). These mice were a kind gift from Dr Tanya Tolmachova of Imperial College London, London, UK.

Rosa26-ERT2-Cre Dicer$^{floxed/floxed}$ (Dicer KO), which are conditional knockout mice for the Dicer gene expression in the presence of tamoxifen were a kind gift from Dr Mark Wilson of National Institute for Medical Research/Francis Crick Institute, London, UK and Genentech, California, United States of America (USA).

2.1.2 Reagent preparations

All following reagents were sterile filtered through 0.22µm pore size hydrophilic, polyethersulpfone membrane, gamma irradiation sterilised and low protein binding filter unit (Merck Millipore, Burlington, Massachusetts, USA) in a class II biological safety cabinet prior to use with cells.

2.2 Mouse cell isolation and culture

2.2.1 Media

2.2.1.1 Mouse complete media

Cells were grown in Roswell Park Memorial Institute (RPMI)-1640 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS) (General Electric (GE) Healthcare, Chicago, Illinois, USA), 100 units/mL penicillin and 100µg/mL streptomycin, 2mM L-glutamine, 10mM HEPES and 50µM 2-mercaptoethanol (all Thermo Fisher Scientific).

2.2.1.2 Mouse EV-free complete media

The same formulation as above 'mouse complete media' but with the heat-inactivated FCS, ultracentrifuged at 100,000g with a Beckman Type 70.1 Ti rotor on a Beckman L8-60M ultracentrifuge machine for 18 hours at 4°C to deplete serum EVs prior to preparing 'mouse EV-free complete media'.

2.2.2 BALB/c mice BM-DCs isolation and culture

Bone marrow was extracted from the tibia, femur and pelvic bones of BALB/c mice using a 27Gx½" needle (BD Biosciences, Franklin Lakes, New Jersey, USA) and a syringe filled with RPMI-1640. The harvested bone marrow was then passed through a 70µm nylon cell strainer (Falcon®, Corning, New York, USA) to gain a single cell suspension. Red blood cells (RBCs) were lysed for 2-5 minutes with ammonium chloride potassium (ACK) lysing buffer (150mM NH_4Cl, 10mM $KHCO_3$ 0.1mM Na_2 EDTA in dH_20; made in-house) at room temperature and cells subsequently washed with RPMI-1640. RBC-lysed cells were incubated with a mixture of 300µL each of rat anti-mouse CD4 (YTS-191), CD8 (YTS-169), MHC class II (M5-114) and B220 (RA3-3A1) purified antibodies (made in-house from hybridoma cell lines) for 30 minutes at 4°C under rotation. Cells were washed in RPMI-1640 to remove any unbound antibodies before incubation with pre-washed Dynabeads™ (magnetic beads pre-coated with polyclonal anti-rat IgGs) (Thermo Fisher Scientific) for 30 minutes at 4°C under rotation. Dynabeads™ which captured contaminating cells and any unbound Dynabeads™ were removed by magnetic

separation using a magnet. The supernatant containing BM-DC progenitor cells were then washed in RPMI-1640 and counted with trypan blue solution (Sigma-Aldrich, St. Louis, Missouri, USA) exclusion of any dead/comprised cells. BM-DC progenitors were then cultured at $1x10^6$ cells/mL/well in 24-well plates with 'mouse complete media' supplemented with 40ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; made in-house; (Tsang et al. 2008)). On days 2 and 4 of culture, non-adherent cells and media were removed then fresh 'mouse complete media' containing 40ng/mL GM-CSF was replenished. On day 6, 'clustering' profile/morphology of BM-DCs was observed under light microscopy before use (**Figure 2.1**). BM-DCs were gamma-irradiated (30 Gray (Gy)) and washed twice with 'mouse complete media' prior to usage for mouse Treg stimulations. For co-culture experiments, the BM-DCs were not irradiated. Cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2.

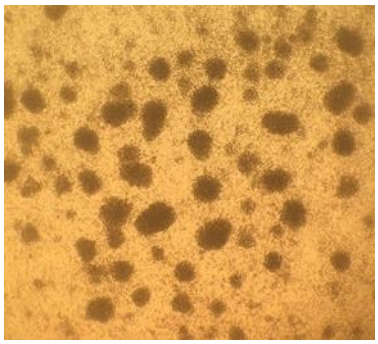


Figure 2.1- BM-DCs 'clustering' morphology profile under light microscopy.

BALB/c BM-DCs were generated from DC progenitors cultured in GM-CSF for 6 days. Representative picture of BM-DC colonies at Day 6 (end of culture) Original objective 10X.

2.2.3 Rosa26-ERT2-Cre Dicer$^{floxed/floxed}$ BM-DC isolation and culture

Bone marrow was harvested, and RBC lysed as described above (**section 2.2.2**). Bone marrow cells were then re-suspended at $10x10^6$/mL in 'mouse complete media' supplemented with 200ng/mL recombinant mouse GM-CSF (Peprotech EC, Hammersmith, London, UK) and with a final concentration of 200nM of 4-hydrotamoxifen (4-OHT) (a kind gift from Dr Mark Wilson of National Institute for Medical Research/Francis Crick Institute, London, UK) or vehicle alone (≥98% ethanol

(Sigma-Aldrich)) in 90mm petri dishes at a total volume of 10mL. On days 2, 5 and 7, the media was changed with fresh media (above formulation) with a final concentration of 200nM of 4-OHT or the equivalent of vehicle alone. On day 10, BM-DCs were harvested for experiments. Cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2. Figure 2.2 shows the Dicer Cre-LoxP system used.

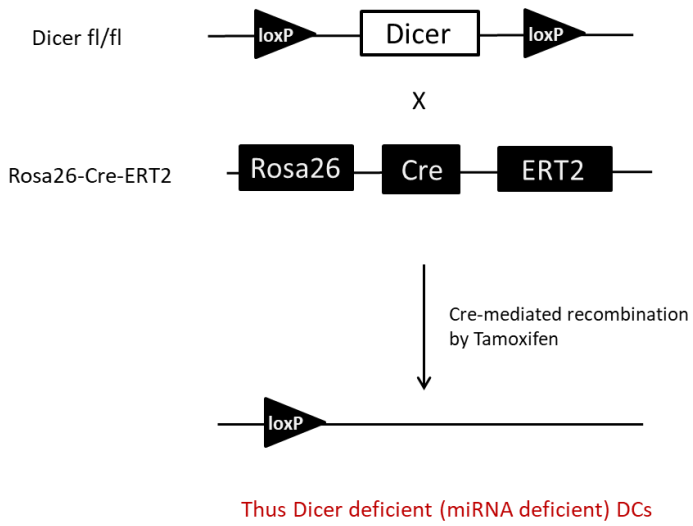


Figure 2.2- Schematic diagram of conditional knock-out of Dicer using Cre-LoxP system

2.2.4 Mouse Treg isolation

The spleen and lymph nodes of B6 and Rab27DKO mice were harvested and $CD4^+CD25^+$ T cells isolated using Dynabeads™ FlowComp™ Mouse $CD4^+CD25^+$ Treg Cells Kit (Thermo Fisher Scientific) according to manufacturer's protocols. Briefly, this protocol involved the negative selection for $CD4^+$ T cells and the subsequent positive selection for $CD25^+$ cells using antibody coated magnetic beads. $CD4^+CD25^+$ Tregs were stimulated with gamma-irradiated (30 Gy) BALB/c BM-DCs at a ratio of 4:1 of Tregs:BM-DCs in 'mouse complete media' in a total volume of 1.5mL in 24-well plates. 10 international units (IU)/mL of recombinant human IL-2 (Proleukin®, Prometheus Laboratories Inc., California, USA) were added to Tregs on days 1, 2 and 4 post-stimulation. Cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2.

2.2.5 Generation of mouse Treg lines

To expand alloantigen-specific mouse Tregs with direct specificity, the B6 Tregs or Rab27DKO Tregs were stimulated weekly with irradiated BALB/c BM-DCs at 4:1 ratio of Tregs:DCs. Similarly, to expand alloantigen-specific mouse T cell with direct specificity, the B6 T cells were stimulated weekly with irradiated BALB/c BM-DCs at 4:1 ratio of T cells:DCs (**Figure 2.3**), in the presence of 10IU/ml IL-2 (Proleukin®) in 24-well plates.

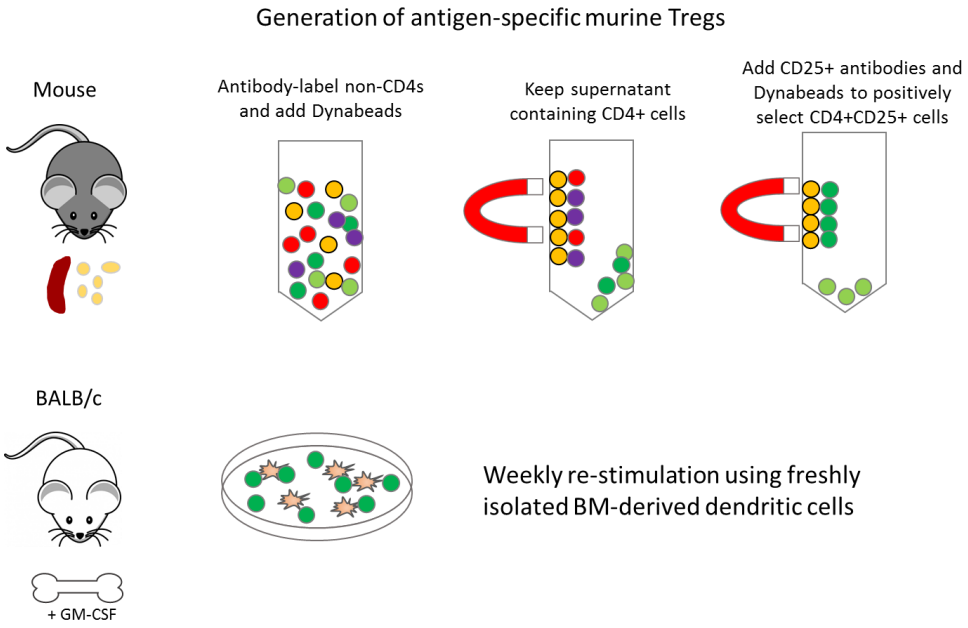


Figure 2.3- Diagram showing the isolation of mouse $CD4^{+}CD25^{+}$ Tregs and expansion of alloantigen-specific Tregs

2.3 Flow cytometry

2.3.1 Phenotypic analysis of mouse cells

All immunostaining was performed in fluorescence-activated cell sorting (FACS) buffer. FACS buffer consisted of 2% FBS and 1mM EDTA in phosphate buffered saline (PBS; all Thermo Fisher Scientific). Staining was performed using 3-4x10^{5} mouse cells per staining panel in a final volume of 100µL of FACS buffer. Cells were incubated with 5µg/mL of αCD16/CD32 antibody (clone 93) (Thermo Fisher Scientific) for 30 minutes at 4°C to block Fc receptors to reduce non-specific antibody binding. The cells were then

incubated with fluorescently-conjugated antibodies specific to the antigens of interest (or respective isotype control) and at the manufacturer's recommended concentrations (Table 2.1) for 30 minutes at 4°C. After the cell surface staining incubation, cells were washed twice with FACS buffer and acquired on BD LSR-Fortessa™ (BD Biosciences) and analysed using FlowJo® software (TreeStar, Ashland, Oregon, USA). Alternatively, if cells required intracellular staining the following steps was performed.

For intracellular staining, cells were fixed and permeablised with FoxP3/Transcription Factor Staining Buffer (Thermo Fisher Scientific) according to manufacturer's protocols. Briefly, cell pellets were incubated with 500µL of fixation/permeablisation buffer for 30 minutes at 4°C and washed twice with permeablisation buffer. Fluorescently-conjugated antibodies (or respective isotype controls) (Table 2.1) were then added to cells in a final volume of 100µL permeablisation buffer and vortexed thoroughly then incubated for 30 minutes at 4°C. Following incubation, cells were washed twice in permeablisation buffer prior to acquisition on BD LSR-Fortessa™ and analysed using FlowJo®.

Localisation	Specificity	Flourochrome	Host species	Isotype	Clone	Manufacturer/Company	Catalougue Number
Extracellular	CD4	FITC	Rat	IgG2a, kappa	RM4-5	eBioscience	11-0042-85
Extracellular	CD11c	PerCP-Cy5.5	Armenian hamster	IgG	N418	eBioscience	45-0114-80
Extracellular	CD25	PE	Rat	IgG1, lambda	PC61.5	eBioscience	12-0251-82
Extracellular	CD39	PE	Rat	IgG2b, kappa	24DMS1	eBioscience	12-0391-82
Extracellular	CD40	PECy7	Rat	IgG2a, kappa	3/23	BioLegend	124621
Extracellular	CD73	PE	Rat	IgG1	eBioTY/11.8	eBioscience	12-0731-82
Extracellular	CD80	BV421	Armenian hamster	IgG	16-10A1	BioLegend	104725
Extracellular	CD86	PE	Rat	IgG2a, kappa	GL1	eBioscience	12-0862-81
Extracellular	MHC I (H-2Kd)	PE	Mouse	IgG2a	SF1-1.1.1	eBioscience	12-5957-82
Extracellular	MHC II (IAd)	FITC	Mouse	IgG3, kappa	39-10-8	Biolegend	115005
Intracellular	CTLA4	APC	Armenian hamster	IgG	UC10-4B9	eBioscience	17-1522-80
Intracellular	FoxP3	APC	Rat	IgG2a, kappa	FJK-16s	eBioscience	17-5773-82

Table 2.1- Summary of the fluorescently-conjugated antibodies used for staining mouse markers

2.4 Mouse Treg suppression assays

2.4.1 $CD4^+$ T responder cell isolation

Spleens of B6 mice were harvested and passed through a 70µm nylon cell strainer (Falcon®, Corning) to gain a single cell suspension and RBCs were lysed. 'Dynabeads™ Untouched™ Mouse CD4 Cells Kit' (Thermo Fisher Scientific) was used according to manufacturer's protocols to isolate $CD4^+$ T responder cells (Tresps). Briefly, cell pellet was re-suspended in 500µL buffer 1 (provided in kit) and 100µL 'CD4 mixture', which

contains a mixture of rat IgGs that bind to mouse $CD8^+$ T cells, B cells, NK cells, monocytes, macrophages, granulocytes, DCs and erythrocytes. The mixture was incubated for 30 minutes at 4°C under rotation. Cells were washed in RPMI-1640 to remove any unbound antibodies before incubation with 1mL pre-washed Dynabeads™ for 30 minutes at 4°C under rotation. Dynabeads™ which captured non $CD4^+$ T cells were removed by magnetic separation. The supernatants containing $CD4^+$ T cells were then washed in RPMI-1640 and counted with trypan blue solution exclusion of any dead/comprised cells.

2.4.2 APC isolation

Mouse splenocytes depleted of RBCs were obtained as described above (section 2.2.4). Remaining cells were incubated with a mixture of 300µL each of rat anti-mouse CD4 (YTS-191) and CD8 (YTS-169) purified antibodies (made in-house) for 30 minutes at 4°C under rotation. Cells were washed in RPMI-1640 before incubation with pre-washed Dynabeads™ (Thermo Fisher Scientific) for 30 minutes at 4°C under rotation. The cells were washed in RPMI-1640 and resuspended in 'mouse complete media'

2.4.3 Suppression assay set up

$CD4^+$ Tresps proliferation was assessed by either ^{3}H-thymidine incorporation or by carboxyfluorescein succinimidyl ester (CFDA-CFSE; Thermo Fisher Scientific) dye dilution. For CFDA-CFSE-based suppression assays, 5µM CFDA-CFSE was added to $CD4^+$ Tresps and incubated at 37°C for 10 minutes. After incubation CFDA-CFSE staining was quenched by addition of 10% FCS in RPMI-1640 for 5 minutes and subsequent centrifugation.

$5x10^4$ CFDA-CFSE labelled B6 $CD4^+$ Tresps were stimulated with $1x10^5$ BALB/c APCs and 1µg/mL of anti-mouse CD3ε antibody (clone 145-2C11; BD Biosciences). This cell mixture was co-cultured with or without $5x10^4$ direct allospecific Tregs, direct allospecific T cells, Rab27DKO Tregs. Co-cultures were set up in a 96-well round bottom plate with a final volume of 200µL in 'mouse complete media'. Cell co-cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2. After 72

hours of incubation, Tresps proliferation was assessed by CFSE dilution and acquired by BD LSR-Fortessa™ and analysed using FlowJo® software (TreeStar).

Alternatively, for ^{3}H-thymidine incorporation-based antigen specific suppression assays, cell co-cultures were set up as described above but without the addition of anti-mouse CD3ε antibody (clone 145-2C11; BD Biosciences). In the final 18 hours of the 72 hours incubation, cells were pulsed with 1µCi ^{3}H-thymidine per well (Amersham GE Healthcare, Buckinghamshire, UK). After the final 18 hours of incubation, well contents were transferred onto glass fibre Printed Filtermat A (PerkinElmer, Waltham, Massachusetts, USA), dried by microwave oven for 2 minutes and filtermats were placed inside sample bags for betaplate™ plastic wallets (PerkinElmer). 15mL of Betaplate Scintillation fluid (PerkinElmer) was added and the plastic wallet sealed. These were then placed into fliter mat holders to measure cell proliferation by using a 1205 Betaplate® liquid scintillation counter (LKB Wallac, Victoria, Australia). Data output is presented as counts per minute (cpm), representing cells in which ^{3}H-thymidine has been incorporated upon cell division.

2.5 Mouse Treg activation and EV isolation

2.5.1 Media

The same formulation as 'mouse complete media' but with the heat-inactivated FCS (GE Healthcare) ultracentrifuged at 100,000g with a Beckman Type 70.1 Ti rotor on a Beckman L8-60M ultracentrifuge machine for 18 hours at 4°C to deplete serum EVs prior to preparing 'mouse EV-free complete media'

2.5.2 Treg Activation and Isolation procedure

150cm^{2}-sized flasks (Falcon®, Corning) were coated with 5µg/mL αCD3 (clone: 145.2C11; BD Biosciences) and 10µg/mL αCD28 (clone: 61109; R&D Systems, Minneapolis, Minnesota, USA) in a total volume of 4mL in PBS, incubated at 37°C for 2 hours or alternatively at 4°C overnight. After the incubation period, the flasks were washed with PBS.

30-50x10^6 mouse Tregs were washed in RPMI-1640 three times before re-suspension in 'mouse EVs-free complete media'. Mouse Tregs were then added to the pre-washed flasks. Cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2 for 24 hours. After 24 hours, cells were assessed for their activation status by staining with anti-mouse anti-CD69 antibody (clone H1.2F3; Thermo Fisher Scientific) and cells were acquired on BD LSR-Fortessa™ (BD Biosciences) and analysed using FlowJo® software (TreeStar).

Supernatant were then collected and centrifuged at 581g for 5 minutes to pellet cells and cellular debris. Cell-free supernatant were then filtered with gentle pressure through hydrophilic, low protein binding, polyethersulfone, gamma sterilised 0.22µm pore-sized filter unit (Merck Millipore) to remove large vesicles such as microparticles but allowing smaller EVs including exosomes (approximately sized between 50-150nm) to pass through (Lesley Ann Smyth et al. 2013).

This EV-containing supernatant were then further purified using two different methods depending on the experimental question at hand and of which is optimal for addressing the research study question. One of these methods employs the gold standard ultracentrifugation (Lesley Ann Smyth et al. 2013). EVs were pelleted at 100,000g using Beckman L8-60M ultracentrifuge with Beckman Type 70.1 Ti rotor for 1.5 hours at 4°C (Clotilde Théry et al. 2006). The supernatant was discarded and EVs pellet washed in PBS before undergoing ultracentrifugation at 100,000g for a further 1.5 hours at 4°C. The EVs pellet was re-suspended in PBS for transmission electron microscopy (section 2.6) and NanoSight LM-10 analysis (section 2.7).

The second method involved the use of the exosome precipitation solution ExoQuick-TC™ (EQ; Systems Biosciences, SBI, Palo Alto, California, USA) (Tung et al. 2018; Okoye et al. 2014). ExoQuick-TC™ was used according to manufacturer's protocols which involved mixing 1mL of ExoQuick-TC™ to every 5mL of conditioned cell culture media (which has cells and cell debris prior removed) and incubation at 4°C overnight. The next day, the mixture was centrifuged at 1500 x g for 30 minutes and the supernatant discarded. EVs pellet was then re-suspended in 'Lysis buffer' (Systems Biosciences, SBI) (see section 2.10 for subsequent exosomal RNA extraction steps). Figure 2.4 shows the approach of EV isolation.

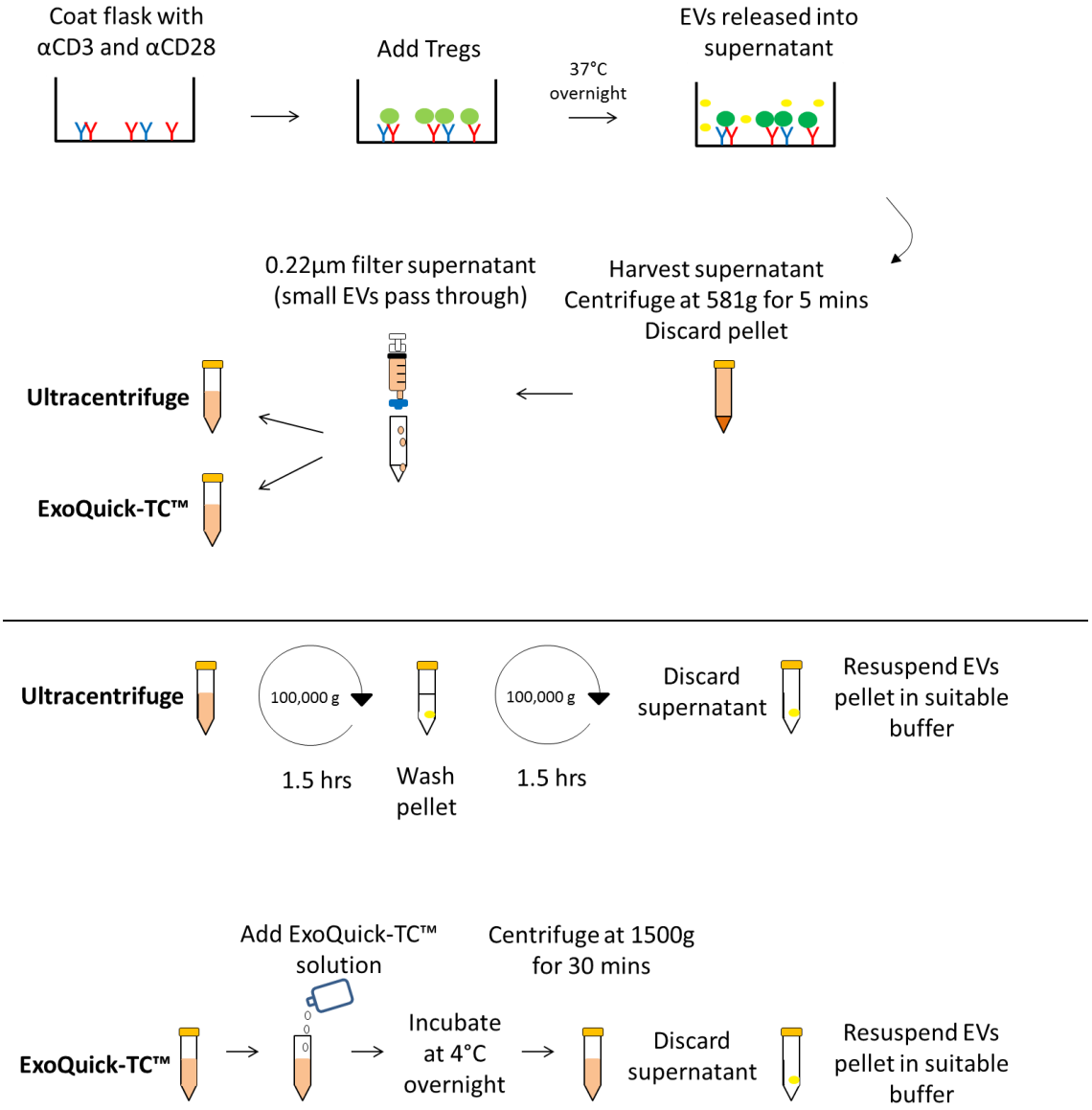


Figure 2.4- Diagram showing the different steps taken to isolate EVs via ultracentrifugation and ExoQuick-TC™.

2.6 Transmission electron microscopy analysis

EVs were isolated using ultracentrifugation and re-suspended in PBS. EVs were added to Formvar/carbon films with copper grids (TAAB Laboratory Equipment Ltd, Aldermaston, Reading, UK) before being fixed with 2% glutaraldehyde (Sigma-Aldrich) in PBS for 5 minutes. The grids were then washed three times, with 2 minutes each, using dH_2O. EVs

were then stained with 3% uranyl acetate and 2% methyl cellulose (both Sigma-Aldrich) in PBS for 20 minutes. Excess fluid was removed using filter papers and grids were air-dried for 5 minutes prior to analysis on transmission electron microscope (FEI Tecnai™ G2 20, Netherlands). All steps were performed at room temperature.

2.7 NanoSight analysis

EVs were isolated using both ultracentrifugation and ExoQuick-TC™ methods and diluted (1:1000) in 0.1µm pre-filtered PBS. 1mL of EV dilutions were taken up in a 1mL syringe and placed into the NanoSight LM10 probe for acquisition. NanoSight LM10 machine was used along with the software nanoparticle tracking analysis (NTA) version 3.2 Dev Build 3.2.16. Settings of SOP Standard Measurement script, laser type Blue 488, sCMOS camera, camera level of 11 and detection threshold of 5 was used consistently in all samples. 30 seconds videos and 3 readings were taken for each sample to gain average measurements of mean, mode, standard deviation, standard error and concentration of particles.

2.8 EVs co-culture with BM-DCs

Mouse Treg EVs were isolated via ExoQuick-TC™ using $40x10^6$ unlabelled or CFSE-labelled Tregs and the unstained EVs or $CFSE^+$ EVs pellet was resuspended in 50µL volume. BALB/c BM-DCs or Rosa26-ERT2-Cre $Dicer^{floxed/floxed}$ BM-DCs were isolated as described above (**sections 2.2.2 and 2.2.3**). $4x10^5$ BM-DCs (harvested on Day 6 of culture) were plated in 48 well plates and $40x10^6$ Treg derived unstained EVs or $CFSE^+$ EVs were added, with a final volume of 400µL in 'mouse complete media'. After 4 hours, 100ng/ml of LPS (Sigma-Aldrich) was added, and after a total of 24 hours of incubation, supernatant of the co-cultures was harvested and stored in -20°C for cytokine analyses. Cell co-cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2.

For uptake of $CFSE^+$ EVs into BM-DCs, the cells were washed two times in cold PBS prior to re-suspension in FACS buffer. Cells were acquired on a BD LSR-Fortessa™ and data analysed using FlowJo® software.

For co-culture of EVs with Rosa26-ERT2-Cre Dicer$^{floxed/floxed}$ BM-DCs miRNA analysis, the cells were washed twice in cold PBS to remove unbound EVs. The cell pellet was the resuspended in 750µL of TRIzol® Reagent (Thermo Fisher Scientific) for RNA extraction (see section 2.9).

2.8.1 Mouse cytokine analysis/Cytometric Bead Array (CBA)

Frozen supernatants from 'EVs co-cultures with BM-DCs' (section 2.8), were thawed and supernatant mixed via pipetting. 'CBA Mouse/Rat Soluble Protein Master Buffer Kit' from BD Biosciences was used to measure cytokine presence and concentrations according to manufacturer's protocols. The BD™ CBA Flex Set Capture beads to detect IL-10, TNF, IL-6 and IL-12 were purchased from BD Biosciences. Briefly, all the capture beads were mixed together and diluted with Capture Bead Diluent (provided in kit) and 50µL of this mixture was added to the assay wells. Subsequently, 50µL of co-culture supernatant was added to the assay wells, mixed and incubated for 1 hour at room temperature. After the incubation period, 50µL of mixed PE Detection Reagent (provided in kit) was added to the assay well, mixed and incubated for 1 hour at room temperature. Assay wells were washed twice with Wash Buffer (provided in kit) before acquisition. Samples were acquired using a BD LSR-Fortessa™ and data analysed using FCAP Array v.3 software (BD Biosciences).

2.9 BM-DC and Treg EVs co-culture RNA extraction

BM-DCs were cultured with or without Treg EVs were used. BM-DCs were washed three times with cold PBS. The cell pellet was resuspended in 750µL of TRIzol® Reagent (Thermo Fisher Scientific) and vortexed for 2 minutes, and subsequently incubated for 5 minutes at room temperature. 150µL chloroform (Sigma-Aldrich) was added to the mixture and vortexed for 15 seconds and incubated for 3 minutes at room temperature. The tubes were then centrifuge at 12,000g for 15 minutes at 4°C. The aqueous phase (top layer) consisting of approximately 400µL was harvested and 1.5 volumes (600µL) of 100% ethanol was added. The mixture was then transferred to RNeasy spin columns (Qiagen, Hilden, Germany) and centrifuged at 8000g for 15 seconds. 500µL of buffer

PRE (provided in RNeasy® Mini Kit, Qiagen) was added to the columns and centrifuged at 8000g for 15 seconds. The columns were then centrifuged at 8000g for 2 minutes to dry them. 30µL of RNase-free water (Qiagen) was added to column membrane and centrifuged at 8000g for 1 minute to elute the RNA. The eluted RNA was stored at -80°C until use.

2.10 Mouse Treg EVs and T cell EVs RNA extraction

30-50x10^6 cell-derived EVs were isolated using ExoQuick-TC™ and the RNA was extracted from EVs using SeraMir™ columns (Systems Biosciences, SBI) according to manufacturer's protocols. Briefly, the EV pellet was resuspended in 350µL Lysis Buffer (provided in kit); vortexed for 15 seconds and incubated for 5 minutes at room temperature. 200µL of 100% ethanol was added and vortexed for 10 seconds. The mixture was transferred to the SeraMir™ columns and collection tube and centrifuged at 16,000g for 1 minute. The column was washed with 400µL Wash Buffer (provided in kit) and centrifuged at 16,000g for 1 minute. The column was centrifuged again at 16,000g for 2 minutes to dry the column. 30µL of RNase-free water was added to column membrane and centrifuged at 380g for 2 minutes to load the membrane with the water then centrifuged at 16,000g for 1 minute to elute the RNA. The eluted RNA was stored at -80°C until use.

2.10.1 DNA removal from RNA

The RNA preparations were treated to remove any contaminating DNA. 50µL RNase-free water, 10µL 10X DNase buffer and 10µL Turbo DNase (all Thermo Fisher Scientific) was added to the eluted RNA. The mixture was incubated at 37°C for 1 hour. Subsequently, an equal volume (100µL) of phenol/chloroform (Sigma-Aldrich) was added and centrifuged at 16,000g for 10 minutes. The aqueous phase was harvested, and 1.5 volumes of 100% ethanol were added. The mixture was passed through RNeasy spin columns and further processed as described above (section 2.9). The eluted DNA-free RNA was stored at -80°C until use.

2.10.2 NanoDrop™ and Bioanalyser

NanoDrop™ spectrophotometer (Thermo Fisher Scientific) and 2100 Agilent Bioanalyzer version 2.6 (Agilent Technologies, Santa Clara, California, USA) with Eurkaryote Total RNA Nano chips (Agilent Technologies) were used to assess RNA concentration and RNA integrity. Both machines were used according to the manufacturer's guidelines.

2.11 Mouse miRNOME screen and real-time PCR validation assays

For the miRNOME screen, reverse transcription was performed using Megaplex™ RT Primers, Pools A v2.1 and Pool B v3.0 with the TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's protocols. The cDNA was then pre-amplified by mixing 7.5µL reverse-transcribed miRNA, 20 µL of TaqMan® PreAmp Master Mix (2X), 8.5 µL of dH_2O and 4 µL of Megaplex™ PreAmp primers Pool A or Pool B (10X) (all Thermo Fisher Scientific). The mixtures were then subjected to the pre-amplification procedure consisting of thermal conditions of 95°C for 10 minutes, 55°C for 2 minutes, 72°C for 2 minutes, then 16 cycles of the following: 95°C for 15 seconds and 60°C for 4 minutes, this was then completed with a final step of 99.9°C for 10 minutes (Pergoli et al. 2017).

The pre-amplified DNA was diluted 1:20 in dH_2O. Equal volume of TaqMan OpenArray® Real Time PCR Master Mix (2X; Thermo Fisher Scientific) was added. 7µL of this mixture was then added with the MicroLab STAR Let instrument (Hamilton Robotics, Birmingham, UK) into the 384-well OpenArray® plate (Thermo Fisher Scientific). The QuantStudio™ AccuFill System Robot (Thermo Fisher Scientific) was used to load the reaction mix from the 394-well OpenArray® plate into a TaqMan™ OpenArray® Human miRNA panel (Pergoli et al. 2017). miRNA was detected using QuantStudio™ 12K Flex Real-Time PCR System and the OpenArray® Platform (QS12KFlex) according to the manufacturer's instructions (Thermo Fisher Scientific). miRNA expression was assessed using the relative quantification 2-ΔCrt method (Livak and Schmittgen 2001).

For miRNOME validation assays and for cellular miRNA detection, cDNA was synthesised using miScript II RT Kit (Qiagen) and real-time PCR assays using QuantiTect SYBR® Green PCR Kits (Qiagen) were performed following manufacturer's instructions. miR-150-5p

(UCUCCCAACCCUUGUACCAGUG), miR-142-3p (UGUAGUGUUUCCUACUUUAUGGA), miR-384-5p (UGUAAACAAUUCCUAGGCAAUGU) and RNU6 primers were purchased from Qiagen. Real-time PCR assays were performed using ViiA™ 7 Real-Time PCR system (Thermo Fisher Scientific). All samples for miRNOME and individual real-time PCR assays was performed using three biological replicates per group, each performed in technical triplicates.

2.12 Human

2.12.1 Hybridoma cell culture

2.12.1.1 Media

'Hybridoma PFHM-II media' were prepared as follows: Gibco® Protein Free Hybridoma Medium (PFHM-II) supplemented with 100 units/mL penicillin, 100µg/mL streptomycin, 10mM HEPES and 50µM 2-mercaptoethanol (all Thermo Fisher Scientific).

'Hybridoma RPMI media' were prepared as follows: RPMI-1640 supplemented with 5% heat-inactivated FCS, 100 units/mL penicillin and 100µg/mL streptomycin (all Thermo Fisher Scientific).

2.12.1.2 Hybridoma cell culture and purification of produced antibody anti-human αCD28.2

αCD28.2 antibody was purified from a hybridoma cell line and using Two-Compartment Bioreactor cell culture flasks (CELLine CL350, Integra Biosciences, Zizers, Switzerland) according to manufacturer's protocols. Briefly, a hybridoma cell line producing αCD28.2 antibody was thawed and cultured in 'hybridoma RPMI media' for 10 days to achieve a log growth phase. 7-8x10^6 cells were resuspended in 5mL of 'Hybridoma PFHM-II' and inoculated in the 'cell compartment' of the Two-Compartment Bioreactor cell culture flask, subsequently 250mL of 'hybridoma RPMI media' was added to the 'medium compartment'. After 1 week of culture, cells were harvested and centrifuged at 300g for 5 minutes. The supernatant containing αCD28.2 antibody was collected and stored at 4°C. The cell pellet was resuspended in 'Hybridoma PFHM-II' and counted. 7-8x10^6 cells were re-seeded into the 'cell compartment' with a fresh supply of 250mL of 'hybridoma

RPMI media' (in the 'medium compartment'. This process was repeated every 3-4 days until 15mL of supernatant containing αCD28.2 antibody was collected. Cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2.

αCD28.2 antibody was concentrated using a three-step filtration system. Supernatant containing the αCD28.2 antibody was filtered using a 0.22µm-sized filter unit. Centriprep® 30K Centrifugal Filter Device (Merck Millipore) was then used to filter contaminants of ≤30 kDa size, retaining molecules of >30kDa size. The filter devices were washed with PBS before use. 15mL of the supernatant was transferred into the 'sample container' part of the device, then the 'filtrate collected' was added. Devices were centrifuged at 1500g for 20 minutes at 4°C. Supernatant that was depleted of ≤30 kDa size molecules was collected from the 'sample container' compartment. The collected supernatant was further filtered using an Amicon® Ultra-15 100K Centrifugal Filter Device (Merck Millipore), which filters molecules <100 kDa size. Supernatant from the previous step was transferred into the filter device and centrifuged at 4000g for 30 minutes at 4°C. Purified antibody was collected from the 'filter device'.

Concentrated αCD28.2 antibody was quantified using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific), according to manufacturer's protocols. Absorbance was measured at 540nm and protein concentration was calculated from a protein standard curve. αCD28.2 antibody concentrations were adjusted to 1mg/mL with sterile PBS, aliquoted and stored at -20°C until use.

2.13 Human cell isolation and culture

2.13.1 Subjects

All human blood and skin samples were obtained from anonymised healthy donors with written informed consent and full ethical approval. Donor peripheral blood contained within leukocyte-enriched blood cones which were provided by National Blood Service (NHS Blood and Transplantation, Tooting, London, UK) with ethical approval from the Institutional Review Board of Guy's Hospital; reference 09/H0707/86. Human skin samples were obtained by abdominoplasty or reduction mammoplasty with ethical

approval from Guy's and St. Thomas' NHS Foundation Trust and King's College London; reference 06/Q0704/18.

2.13.2 Media

'Human complete media' were prepared as follows: X-VIVO™ 15 (Lonza, Basel, Switzerland) supplemented with 5% heat-inactivated human serum (HS) (Biowest, Riverside, Missouri, USA).

'Human EVs-free complete media' were prepared as follows: X-VIVO™ 15 (Lonza) supplemented with 5% heat-inactivated FCS (Thermo Fisher Scientific) previously ultracentrifuged at 100,000g with a Beckman Type 70.1 Ti rotor on a Beckman L8-60M ultracentrifuge machine for 18 hours at 4°C to deplete serum EVs prior to preparing 'human EVs-free complete media'.

2.13.3 Human peripheral blood mononuclear cells (PBMCs) isolation

Human PBMCs were isolated from the blood of healthy donors. Leukocyte-enriched blood cones were diluted 8-fold with PBS prior to carefully layering it over 15mL of the density gradient medium Lymphoprep™ (StemCell™ Technologies, Vancouver, British Columbia, Canada). This was then centrifuged at 940 x g for 20 minutes with no brake. The buffy coat containing total PBMCs present at the plasma:Lymphoprep™ interface was collected and washed 3 times in PBS to remove any contaminate platelets. Total PBMCs were cryopreserved in cell freezing medium (consisting of 90% heat-inactivated FCS and 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich)) and frozen in -80°C for 24 hours prior to immediate storage in liquid nitrogen until use. For $CD25^-$ PBMCs collection, the total PBMCs population were then depleted of Tregs by the positive selection of $CD25^+$ cells. Total PBMCs were mixed with CD25 MicroBeads II (Miltenyi Biotec, Cologne, Germany) and incubated at 4°C under rotation for 15 minutes. Cells were then washed to remove excess CD25 MicroBeads II and re-suspended in 3mL MACS buffer (consisting of 0.5% BSA and 2mM EDTA in PBS). Cells were passed through a pre-primed 50µm pre-separation filter, LS column and a MACS separator magnet (all Miltenyi Biotec). The eluent containing $CD25^-$ PBMCs were collected. The $CD25^-$ PBMCs

were cryopreserved in cell freezing medium and frozen in -80°C for 24 hours prior to immediate storage in liquid nitrogen until use.

2.13.4 Human $CD4^+$ $CD25^+$ Treg and $CD4^+$ $CD25^-$ conventional T effector cell (Teffs) isolation and culture

Human $CD4^+CD25^+$ Tregs and autologous $CD4^+CD25^-$ Teffs were isolated from the blood of healthy donors. This process involved a negative selection for $CD4^+$ T cells and the subsequent positive selection for $CD25^+$ cells for Tregs.

Leukocyte-enriched blood cones were diluted 2-fold with PBS and incubated with Rosette Sep™ Human $CD4^+$ T cell Enrichment Cocktail (StemCell™ Technologies) (150µL of this was used for every 5mL of blood derived from the blood cone) for 30 minutes at room temperature. This process allowed for non-$CD4^+$ cells, namely cells which expressed CD8, CD16, CD19, CD36, CD56, CD66b or TCRγ/δ to be labelled and crosslinked to red blood cells. After the incubation period, the blood was further diluted by 4-fold with sterile PBS and carefully layered on top of 15mL of Lymphoprep™ (StemCell™ Technologies) and centrifuged at 940g for 20 minutes with no brake. The buffy coat containing $CD4^+$ T cells were collected and CD25 MicroBeads II (Miltenyi Biotec) was used to separate $CD4^+$ $CD25^+$ Tregs and $CD4^+$ $CD25^-$ Teffs as mentioned above. $CD4^+$ $CD25^+$ Tregs were collected by removing the LS columns holding the $CD25^+$ cells from the MACS separator magnet and eluting them by gentle force with the plunger. The purity of $CD4^+$ $CD25^+$ cells were >94% as tested by immunostaining cells immediately following isolation with CD4 (clone OKT4; Thermo Fisher Scientific) and CD25 (clone 2A3; BD Biosciences) (**Figure 4.1**).

Purified $CD4^+CD25^+$ T cells were activated using Dynabeads® Human T-Activator CD3/CD28 for T Cell Expansion and Activation (αCD3/CD28 beads; Thermo Fisher Scientific) at a 1:1 ratio of cells:beads. $CD4^+CD25^+$ T cells were added at $1x10^6$ cells/mL per well of a 24-well plate in the presence of 100nM rapamycin (LC-Laboratories, Woburn, Massachusetts, USA) using human complete media.

1000 international units (IU) of IL-2/mL and 100nM rapamycin in 'human complete media' was added to $CD4^+CD25^+$ T cells every second day thereafter. Human Tregs were re-stimulated on day 10, this was done by removing remaining αCD3/CD28 beads via

magnetic separation and adding cells to 'human complete media' containing αCD3/CD28 beads, rapamycin and IL-2 at the above concentrations. Suppressive capacity and phenotype were tested after 2 stimulations and cells were used for experiments after confirmation of their suppressive function (approximately day 20-22 of culture).

Purified $CD4^+CD25^-$ T cells were activated using Dynabeads® Human T-Activator CD3/CD28 for T Cell Expansion and Activation (αCD3/CD28 beads) at a 1:1 ratio of cells:beads. $CD4^+CD25^-$ T cells (Teffs) were added at $1x10^6$ cells/mL per well of a 24-well plate. Teffs received 100IU/mL of recombinant IL-2 every 2 days. Cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Remaining freshly isolated $CD4^+CD25^-$ T cells were cryopreserved in cell freezing medium and used later as Tresps for 'human suppression assays' (section 2.16).

2.14 Flow cytometry

2.14.1 Phenotypic analysis of human cells

All immunostaining was performed in FACS buffer using $2\text{-}4x10^5$ human cells per staining panel in a final volume of 100µL. The cells were than incubated with fluorescently-conjugated antibodies specific to the antigens of interest (or respective isotype control) and at the manufacturer's recommended concentrations (Table 2.2) for 30 minutes at 4°C.

For intracellular staining, cells were fixed and permeablised with FoxP3/Transcription Factor Staining Buffer (Thermo Fisher Scientific) according to manufacturer's protocols. Briefly, cell pellets were incubated with 500µL of fixation/permeablisation buffer for 30 minutes at 4°C and washed twice with permeablisation buffer. Fluorescently-conjugated antibodies (or respective isotype controls) were then added to cells in a final volume of 100µL permeablisation buffer and vortexed thoroughly then incubated for 30 minutes at 4°C. Following incubation, cells were washed twice in permeablisation buffer prior to acquisition on BD LSR-Fortessa™ and analysed using FlowJo® software.

Localisation	Specificity	Flourochrome	Host species	Isotype	Clone	Manufacturer/Company	Catalougue Number
Extracellular	CD3	APC-H7	Mouse	IgG1, kappa	SK7	BD Biosciences	560275
Extracellular	TCRαβ	APC	Mouse	IgM, kappa	T10B9.1A-31	BD Biosciences	563826
Extracellular	CD4	PerCP-Cy5.5	Mouse	IgG2b, kappa	OKT4	eBioscience	45-0048-42
Extracellular	CD25	BV605	Mouse	IgG1, kappa	2A3	BD Biosciences	562661
Extracellular	CD127	FITC	Mouse	IgG1, kappa	eBioRDR5	eBioscience	11-1278-42
Extracellular	CD39	PE-Cy7	Mouse	IgG1, kappa	eBioA1	eBioscience	25-0399-42
Extracellular	CD73	PE	Mouse	IgG1, kappa	AD2	eBioscience	12-0739-42
Extracellular	CD69	PerCP	Mouse	IgG1, kappa	FN50	BioLegend	310928
Extracellular	CD62L	BV605	Mouse	IgG1, kappa	DREG-56	BD Biosciences	562719
Extracellular	CCR4	BV605	Mouse	IgG1, kappa	L291H4	BioLegend	359418
Extracellular	CD45	PE	Mouse	IgG1, kappa	2D1	eBioscience	12-9459-42
Extracellular/Intracellular	CD63	PE	Mouse	IgG1, kappa	H5C6	eBioscience	12-0639-42
Extracellular/Intracellular	CD63	FITC	Mouse	IgG1, kappa	H5C6	BD Biosciences	561924
Extracellular/Intracellular	CD81	PE-Cy7	Mouse	IgG1, kappa	5A6	BioLegend	349512
Extracellular/Intracellular	CD9	PE	Mouse	IgG1	eBioSN4	eBioscience	12-0098-42
Intracellular	FoxP3	eFluor 660	Rat	IgG2a, kappa	PCH101	eBioscience	50-4776-42
Intracellular	CTLA4	BV421	Mouse	IgG2a, kappa	BNI3	BD Biosciences	562743

Table 2.2- Summary of the fluorescently-conjugated antibodies used for staining human markers

2.15 Human Treg and Teffs activation and EV isolation

At day 20 post human Treg isolation, Tregs and Teffs were rested by depletion of activation beads and culture media and Tregs were given low-dose IL-2 at 100IU/mL only (no addition of rapamycin), Teffs were given 10IU/mL of IL-2. Cells were incubated for 48 hours before activation for EV release. 12-well TC plates were coated with 5µg/ml αCD3 (clone: OKT3; Thermo Fisher Scientific) and 10µg/ml αCD28 (clone: αCD28.2) in a total volume of 0.5mL in PBS, incubated at 37°C for 2 hours or alternatively at 4°C overnight. After the incubation period, the wells were washed with PBS. Human Tregs or Teffs were washed three times in X-VIVO™ 15 before re-suspension in 'human EVs -free complete media', no addition of IL-2 or rapamycin was added.

$3x10^6$ human Tregs or Teffs were then added to the pre-washed wells at a volume of 1mL per well. Cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2 for 24 hours. After 24 hours, cells were assessed for their activation status by CD69 expression levels by flow cytometry analysis.

Supernatant were then collected, and differential centrifugation was performed as previously described (Clotilde Théry et al. 2006). The differential centrifugation involved the supernatant to be centrifuged at 300g for 10 minutes to pellet cells and dead cells. The supernatant from the previous step was collected and centrifuged at 2000g for 10

minutes to remove cellular debris. The supernatant from the preceding step was collected and ultra-filtered with gentle pressure through a 0.22µm pore-sized filter to remove large vesicles. This EVs-rich supernatant was then further purified using two different methods as described above for ultracentrifuge and ExoQuick-TC™.

EV isolation via ultracentrifuge was employed for transmission electron microscopy, NanoSight LM-10 analysis and phenotyping of EVs; EV pellet was re-suspended in PBS. Whereas, EV isolation via ExoQuick-TC™ was employed for EV RNA extraction, EV suppression assays with subsequent cytokine bead array analysis and for the *in vivo* experiments. EVs derived from ExoQuick-TC™ was re-suspended in RLT buffer (Qiagen) for RNA extraction; 'human EVs-free complete media' for EV suppression assays with cytokine bead arrays analysis; and sodium chloride (NaCl) 0.9% w/v saline (B Braun, Sheffield, UK) for *in vivo* analysis.

2.15.1 Phenotypical analysis of EVs

For phenotyping Treg EVs, EVs were isolated using ultracentrifugation. $50x10^6$ human Tregs were used per EV phenotyping experiments. The EV pellets were resuspended in a total volume of 50µL of PBS. The EVs were incubated with 4µm aldehyde/sulphate latex beads (Thermo Fisher Scientific) for 15 minutes to allow EVs to adhere to the latex beads. 10µg bovine serum albumin (BSA) (Sigma-Aldrich) in PBS was added to latex beads+ EVs and the mixture was incubated under rotation at room temperature for 75 minutes to block non-specific binding areas. EVs/latex beads were washed in PBS and centrifuged at 300g for 5 minutes. 100mM glycine (Sigma-Aldrich) was mixed with the EVs/latex beads pellet and incubated for 30 minutes at room temperature. EVs/latex beads were washed three times in 3% FCS in PBS (Lesley Ann Smyth et al. 2013).

The EVs/latex beads pellet was resuspended in 100µL in 3% FCS in PBS. 5µLs of fluorochrome-conjugated antibodies (Table 2.2) were added to the EVs/latex beads mixture and incubated for 30 minutes at 4°C. After incubation, EVs/latex beads were washed twice in 3% FCS in PBS before acquisition on BD LSR-Fortessa™ and analysed using FlowJo® software.

2.16 Human cell and EVs CTV-based suppression assays

The suppressive capacity of human Tregs and Teffs were assessed after 20-22 days of expansion. Previously frozen $CD4^+CD25^-$ Tresps autologous to the Tregs and Teffs were thawed. Tresps were washed twice in PBS. Next, Tresps were incubated with 2µM CellTrace Violet (CTV) (Thermo Fisher Scientific) for 20 minutes at 37°C. The labelling reaction was quenched with 'human complete media' and incubated for 5 minutes. Cells were washed twice in 'human complete media'. Human Tregs were harvested and remaining αCD3/CD28 beads removed by using a magnet. Cells were washed and resuspended in 'human complete media'.

$1x10^5$ CTV-labelled autologous $CD4^+$ Tresps were stimulated with αCD3/CD28 beads at a 1:40 ratio of bead:Tresps in a volume of 100µL plated in a 96-well round bottom plate. Varying numbers of Tregs at indicated ratios were added, and final volume of 200µL per well was achieved by addition of 'human complete media'. Alternatively, Treg EVs or Teff EVs isolated via ExoQuick-TC™ and derived from autologous 5, 10, 35 or $50x10^6$ Treg or Teff cells were added.

Cells/EVs co-cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2. After 5 days of incubation, co-cultures were pelleted and supernatant harvested and stored at -20°C for cytokine analyses. Tresp proliferation was assessed by CTV dilution by acquisition on a BD LSR-Fortessa™ and data analysed using FlowJo® software.

2.16.1 Human CBA assays

Frozen supernatant from the human cell/EV co-cultures suppression assays (**section 2.16**) were defrosted and mixed via pipetting. Cytokine presence was measured using Human Th1/Th2/Th17 Kit BD™ Cytometric Bead Array kit (BD Biosciences). Briefly, 10µL of mixed Capture Beads were added to V-bottom 96 well culture plates (CellStar®, Greiner Bio-One, Kremsmunster, Austria). 50µL of co-culture supernatant and 10µL of Human Th1/Th2/Th17 PE Detection Regent was then added. Mixtures were incubated for 3 hours at room temperature in the dark. To wash samples, 100µL of Wash Buffer were added to the wells and centrifuged at 200g. Supernatant were aspirated and

200µL of Wash Buffer were added to the wells and centrifuged at 200g. The supernatant was aspirated, and capture beads pellet was resuspended in 300µL of Wash Buffer. Cytokine standards were prepared using the supplied kit reagents. Samples were acquired on a BD LSR-Fortessa™ and data analysed using FCAP Array v.3 software.

2.17 Human Tresps co-culture with CFSE$^+$ Treg EVs

Human Treg EVs were isolated via ExoQuick-TC™ using 50x10^6 CFSE-labelled Tregs (section 2.4.3 for CFDA-CFSE labelling procedure) and the CFSE$^+$ EVs pellet was resuspended in 50µL volume of 'human complete media'.

Human autologous Tresps were defrosted from frozen stocks. 5x10^5 Tresps were plated in 48-well plates and 50x10^6 Treg derived CFSE$^+$ EVs were added, with a final volume of 500µL in 'human complete media'. Cells/EVs co-cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2 for 24 hours. Tresps were washed thoroughly twice in cold PBS prior to re-suspension In FACS buffer. Cells were acquired on a BD LSR-Fortessa™ and data analysed using FlowJo® software.

2.18 Human cell and EVs RNA extraction

2x10^6 resting human Tregs or the autologous resting Teffs were washed in PBS and resuspended in 700µL RLT buffer (Qiagen) containing 1% 2 β-mercaptoethanol (Sigma-Aldrich) and vortexed to complete homogenises (Eldh et al. 2012; Okoye et al. 2014). 50x10^6 autologous Treg EVs or autologous Teff EVs were isolated using the ExoQuick-TC™ method and the EVs pellet was resuspended in 700µL RLT buffer containing 1% 2 β-mercaptoethanol and vortexed. All cells and EVs preparation were then stored in -80°C until RNA isolation. Samples were thawed and vortexed, 3.5 times the ethanol was added and mixed by pipetting. The samples were then passed through RNeasy spin columns and the columns were subsequently washed twice with RPE buffer (Qiagen), and the membrane dried by brief centrifugation. 30µL of RNase-free water was added to the membrane and RNA eluted by centrifugation. Purified RNA was stored in -80°C.

2.19 Human cell and EVs miRNOME screen and real-time PCR validation assays

RNA samples were shipped to Exiqon and a complete miRNA analysis (miRNOME) was undertaken. Briefly, according to the company, miRNA was polyadenylated and reverse transcribed into cDNA. cDNA and ExiLENT SYBR® Green master mix were transferred into miRCURY LNA™ Universal RT microRNA PCR Human panels I+II (all Qiagen), which were preloaded with primers. Amplification was performed using a Roche Lightcycler 480. Global mean normalisation was used as the normalisation method for the miRNOME as this was the most stable normaliser for this screen. Validation of the human miRNOME results was performed by individual qPCR assays as described above (section 2.11).

2.20 Bioinformatics

Bioinformatic tool microRNA.org (www.microrna.org) was used to map the 3'-UTR of IFNγ, IL-2 and IL-6 mRNAs and to *in silico* predict their associated miRNA targets, along with the algorithms of mirSVR and PhastCons scores. Another bioinformatic tool mirTAR (mirtar.mbc.nctu.edu.tw/human/) was used to *in silico* predict miRNAs to target multiple genes in KEGG pathways.

2.21 Humanised mouse skin transplant *in vivo* (xenograft) model

Human skin samples were donated by healthy donors, who had abdominoplasty or reduction mammoplasty surgery. Human skin was transplanted onto mice within 24 hours from the time of skin explant from patients. The skin was disinfected with 100units/mL penicillin and 100µg/mL streptomycin in PBS. Split-thickness skin explants were processed using an electrical dermatone using firm pressure to obtain a uniform 0.5-0.7mm thickness of the upper layers of the donor human skin which included the epidermal and dermal layers. Skin explants were cut into 1.5cm^2 grafts using a sterile scalpel and kept moist by wrapping skin explants in sterile gauze swabs that were pre-soaked with 100 units/mL penicillin and 100µg/mL streptomycin in PBS and stored at 4°C until commencement of transplantation procedure.

Transplantation of human skin grafts onto 10-12 weeks old BRG mice were performed as previously described (P. Sagoo et al. 2011; Putnam et al. 2013). Mice were injected with 2µg Vetergesic Multidose analgesia (Alstoe Ltd, Laval, France) by the subcutaneous route and put under general anaesthesia using Isoflurane (Merial, Sandringham, UK). A small area of skin on the dorsal (back) of the mice were shaved and disinfected with Vetasept povidone-iodine antiseptic solution (Animalcare Ltd., York, UK). Subsequently, a 1.5cm^2-sized mouse skin excision was made and replaced with the human skin graft. The human skin graft was adhered onto the mouse skin using Vetbond™ Tissue Adhesive (3M™, Maplewood, Minnesota, USA) and Fucidin® fusidic acid cream (LEO Pharma, Ballerup, Denmark) was applied liberally over the allograft and adjoining edges of mouse and human skin. A three-layer dressing was applied to mice which consisted of the first layer of OpSite® Spray Dressing (Smith & Nephew plc, London, UK); the second layer of Jelonet paraffin gauze dressing (Smith & Nephew plc); and the third layer of Tegaderm™ Transparent Film Dressing (3M™), which was wrapped around the mid-body section of the mice to secure the allograft and above components.

Mice were checked weekly and allografts were allowed to engraft for 5-6 weeks, after which the mice were intravenously (via the tail vein) injected with saline (B Braun) or 5x10^6 CD25$^-$ PBMCs ± 1x10^6 Tregs or ± 50x10^6 Treg EVs or ± 50x10^6 Teff EVs or ± 'media pellet'. 50x10^6 Treg EVs were isolated either by ultracentrifugation or ExoQuick-TC™. 50x10^6 Teff EVs and 'media alone pellet' were isolated using ExoQuick-TC™. Total injection volume was 300µL and saline was used as the resuspension buffer for all cells and EVs. Anti-Gr1 injections were administered bi-weekly via intraperitoneal route. Mice were monitored twice weekly to check their general health and to detect any changes in skin allograft morphology and mice were weighed twice weekly to detect for signs of xeno-GvHD. At week 4, a sample of peripheral blood was collected via the tail vein to assess human immune cell engraftment by immunostaining for human and mouse CD45$^+$ cell and acquired by flow cytometry. 5 weeks post cell ± EVs injection; mice were culled by CO_2 over administration and neck dissociation. Photographs of mice with the human skin allografts were taken on a 12-megapixel camera using natural light settings on a white background. Human skin allografts with a small surrounding area of mouse skin were carefully excised for histological analysis. Spleens were collected for measuring human CD45$^+$ cell engraftment.

2.22 Histology preparation of slides

After excision of the transplanted human skin, the skin explant was placed flat inside Peel-A-Way® disposable embedding molds (histomold) (Polysciences Inc., Pennsylvania, USA) containing optimal cutting temperature (OCT) compound (Thermo Fisher Scientific) and frozen over dry ice. Skin sections were cut using a cryostat (Leica Biosystems, Wetzlar, Germany) at a thickness of 8µm, mounted onto poly-L-lysine adhesion slides (Thermo Fisher Scientific) and air dried for 2 hours at room temperature in the dark before storage in -80°C.

2.22.1 H&E staining

Slides containing skin sections were removed from -80°C and left to air dry for 10 minutes before being fixed with 10% formalin (Thermo Fisher Scientific) for 5 minutes and washed with running tap water for 1 minute. To stain the nuclei, skin sections were immersed in haematoxylin solution, gill number 3 (Sigma-Aldrich) for 1 minute. To remove excess haematoxylin, slides were placed under running tap water for 1-3 minutes until the desired stain intensity. Skin sections were placed in 1% acid alcohol for 5-8 seconds to remove cytoplasmic staining (differentiation) and subsequently placed under running tap water. Skin sections were then placed in 1% eosin (Sigma-Aldrich) for 20-30 seconds and washed under running tap water for 10 seconds. Slides were then dehydrated in 70% alcohol twice using different changes of solution, and then slides were placed in 100% alcohol for 2 minutes. Subsequently, the slides were placed in two changes of xylene (Sigma-Aldrich) to remove the alcohol. 2-3 drops of DPX mount solution (Sigma-Aldrich) was added to slides and a clear 1.5mm thickness coverslip was placed on top and allowed to air dry.

2.22.2 Confocal immunostaining and microscopy

Slides containing skin sections were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes at room temperature. Sections were then washed 3 times with PBS for 10 minutes and with a gentle rocking motion. Slides were blotted with tissue paper closely around the sections and using a DAKO pen (Agilent Technologies), a circle was drawn

around the section to create a hydrophobic barrier to enclose the sections. Blocking solution consisting of 10% goat serum, 0.1% fish skin gelatin, 0.1% Triton™ X-100 and 0.5% Tween® 20 (all Sigma-Aldrich) in PBS were added to sections and incubated for 1 hour at room temperature. After incubation, the slides were tilted and excess blocking solution blotted on paper towels. Whilst the sections were still moist, the primary antibodies mixture (Table 2.3) were added and sections incubated overnight at 4°C, in the dark. Sections were then washed twice in PBS for 10 minutes each time and with a gentle rocking motion. Secondary antibodies and DAPI (Table 2.3) were added to the sections and incubated for 1 hour at room temperature, in the dark. Sections were washed 3 times in PBS for 5 minutes each time and with a gentle rocking motion. Sections were mounted with Fluorescence Mounting Medium (Agilent Technologies) and a 1.5mm thick clear coverslip was placed on top and slides were stored at 4°C and in the dark until acquisition. Images were taken on a Tei Nikon inverted confocal microscope (Nikon, Minato, Tokyo, Japan), using a 20x objective lens. A large image consisting of 4 fields of view with a 2x2 orientation was taken with the edges blended to create a single large image. Maximum intensity projection (MaxIP) images consisting of 5 z-stacks (1.1µm apart) were acquired. Image analysis was performed on Nikon Instrument Software (NIS) Elements and quantification analysis was performed on Fiji is just ImageJ (FIJI) imaging software using consistent settings.

Layer	Specificity	Flourochrome	Host species	Isotype	Clone	Manufacturer/Company	Catalougue Number
Primary	CD45	N/A- purified	Mouse	IgG1, kappa	HI30	eBioscience	14-0459-82
	CD3	N/A- purified	Rabbit	IgG	polyclonal	Dako	A045229-2
	CD31	N/A- purified	Rabbit	IgG	polyclonal	Abcam	ab28364
	Involucrin	N/A- purified	Mouse	IgG1	SY5	Sigma Aldrich	I9018
	Ki67	N/A- purified	Rabbit	IgG	polyclonal	Abcam	ab15580
Secondary	Goat-anti mouse	AF555	Goat	IgG	polyclonal	Invitrogen	A-21422
	Goat-anti rabbit	AF647	Goat	IgG	polyclonal	Invitrogen	A-21244
Nuclear	DAPI	N/A	N/A	N/A	N/A	Sigma Aldrich	D9542

Table 2.3- Summary of the primary and secondary antibodies and DAPI used for staining human skin allograft

2.23 Imaging EVs

$50x10^6$ Treg cell derived EVs were isolated using ExoQuick™. The EV pellet was resuspended in 90µL of PBS. 1µM CFDA-CFSE was added to the EV pellet. 2µL of anti-human CD63- PE (clone H5C6; Table 2.2) was added and mixture was vortexed for 10

seconds. Samples were then incubated for 45 minutes at 37°C. Samples were centrifuged at 16,000g for 1 minute. EVs were assessed on Amnis ImageStreamX Mark II (Leica Biosystems, Wetzlar, Germany).

2.24 Lentiviral particle production

2.24.1 Media

'Dulbecco's Modified Eagle medium (DMEM) complete media' were prepared as follows: DMEM- low glucose (1000mg/L glucose) (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 100 units/mL penicillin and 100µg/mL streptomycin and 2mM L-glutamine (all Thermo Fisher Scientific).

'DMEM serum-free media' were prepared as follows: DMEM- low glucose (1000mg/L glucose) (Sigma-Aldrich) supplemented with 2mM L-glutamine.

2.24.2 HEK293Ts

Human embryonic kidney (HEK) 293T cells were a kind gift from Dr Gilbert Fruhwirth (Division of Imaging Sciences and Biomedical Engineering, King's College London, London, UK). HEK293T cells were maintained in 'DMEM complete media' and passaged when cell cultures reached 80-90% confluency. To harvest these adherent cells, cells were washed in PBS and incubated with 0.05% Trypsin-EDTA (Thermo Fisher Scientific) for 4-5 minutes at room temperature before harvesting detached cells. Washed cells were counted and re-seeded at optimal concentrations for further expansion for a maximum of 10 passages. Cell cultures were maintained in a humidified incubator at 37°C in the presence of 5% (v/v) CO_2.

2.24.3 Plasmids/constructs

The eGFP-CD63 and mCherry-CD63 plasmids were a kind gift from Professor Tony Ng (Cancer Cell Biology & Imaging, King's College London). pMDLg/pRRE and pRSV-Rev (packaging plasmids) and pCMV-VSV-G (envelope plasmids) were a kind gift from Dr Gilbert Fruhwirth (Division of Imaging Sciences and Biomedical Engineering, King's

College London, London, UK). eGFP-CD63 and mCherry-CD63 plasmid maps are included in Appendix 1 and 2, respectively.

2.24.4 Bacterial transformation of competent cells

Plasmid DNA was reproduced by transforming One Shot® Stbl3™ chemically competent Escherichia coli cells (Thermo Fisher Scientific). Cells were thawed on ice and 100ng plasmid DNA in <2.5µL volumes (typically 1µL) were added to the vial of 25µL of cells. The tube was tapped gently to mix the components and incubated on ice for 30 minutes. The cells were then heat-shocked in a 42°C water bath for 45 seconds and immediately placed on ice for 2 minutes. 250µL of pre-warmed super optimal broth with catabolite repression (SOC) media was added to the vial. Vials were placed in a shaking 37°C incubator. 25µL and 80µL of the transformed cells were spread on pre-warmed Lysogeny broth (LB)-agar (Sigma Aldrich) plates containing 100µg/mL ampicillin (Sigma Aldrich) and incubated at 37°C overnight. The next day, single bacterial colonies were picked using a sterile pipette tip and placed in 2mL L-broth medium containing 100µg/mL ampicillin and incubated for 12-16 hours at 37°C with shaking conditions. A glycerol stock was prepared by adding 50% glycerol (in dH_20) with equal volume of transformed cells mixture (typically 200µL total volume was prepared). Glycerol/cells stocks were stored in -20°C. Remaining cells were further processed for Miniprep DNA purification.

2.24.5 Miniprep DNA purification preparations

Wizard® *Plus* SV Minipreps DNA purification system (Promega, Madison, Wisconsin, USA) were used according to manufacturer's protocols. The first step involved centrifuging the transformed cells at 4000g for 10 minutes at 4°C to pellet the cells. 250µL of Cell Resuspension Solution was then used to resuspend the cell pellet. 250µL of Cell Lysis Solution was added and the tube was inverted 4 times to mix thoroughly. 10µL of Alkaline Protease Solution was added and the tubes were inverted 4 times to mix and incubated for 5 minutes at room temperature. To neutralise the reaction, 350µL of Neutralisation Solution was added and the tube inverted 4 times. The tubes were then centrifuged at 12,000g for 10 minutes at room temperature. The

supernatants, which were the cleared lysate, were then transferred into the Spin Columns held in place by the Collection Tube. The column and tubes were centrifuged at 12,000g for 1 minute and the flow-through was discarded. To wash the column and DNA, 750µL of Wash Solution was added to the column, this was then centrifuged at 12,000g for 1 minute and the flow-through discarded. This wash step was repeated but with 250µL of Wash Solution. To dry spin column, this was centrifuged at 12,000g for 2 minutes. The spin column was then transferred into a new collection tube and centrifuged at 12,000g for 1 minute to drain any residual solution. The spin column was then transferred into a 1.5mL Eppendorf tube and 50µL of nuclease-free dH_2O was added to the membrane of the spin column and centrifuged at 12,000g for 1 minute. To increase the yield of DNA, the eluate was transferred back into the same column membrane and centrifuged again at 12,000g for 1 minute. The eluted DNA was stored at -20°C.

2.24.6 Restriction enzyme digest of plasmid

Enzymes EcoR1-HF® and Age1-HF® (New England BioLabs, Ipswich, Massachusetts, USA) were selected as the diagnostic restriction enzymes to test that the eGFP -CD63 and mCherry -CD63 plasmids were of the correct size. 0.5µL of EcoR1-HF® (= 10 units) and 0.5µL Age1-HF® (= 10 units) enzymes (both New England BioLabs) were added to 400ng of plasmid DNA with CutSmart® buffer (10X) (New England BioLabs) and nuclease-free dH_2O to a final volume of 20µL. The mixture was incubated at 37°C for 1 hour. The DNA was added into a 0.7% agarose (Sigma-Aldrich) electrophoresis gel prepared in Tris-acetate-EDTA (TAE) buffer (Thermo Fisher Scientific) and containing GelRed® nucleic acid gel stain (10,000X) (Biotium, California, USA) at the manufacturer's recommended volume of 10µL per 100mL of gel mixture. 10µL (at the concentration of 100µg/mL) of Quick-Load® 2-Log 0.1-10kb DNA ladder (New England BioLabs) was added to the well lanes as a reference for different DNA sizes. The digested DNA product (20µL) was mixed with DNA Gel loading purple dye (6X) (New England BioLabs) before loading into the well lanes. Gel electrophoresis was conducted at 80 volts for 90-120 minutes before visualisation under a GelDOC-It® Imager UV transilluminator (UVP, California, USA). After confirmation that a selected bacterial clone had taken up the plasmid and is of the

correct orientation, as assessed by restriction enzyme digest size products, this clone was selected for Maxiprep DNA purification.

2.24.7 Maxiprep DNA purification preparations

To prepare cells for Maxipreps, the pre-made glycerol stocks of transformed cells were defrosted and 50µL of the transformed cells/glycerol stocks were added to 200mL of L-broth medium containing 100µg/mL ampicillin. This was incubated for 12-16 hours at 37°C with shaking conditions.

A Plasmid Maxi Kit (Qiagen) was used according to manufacturer's protocols. This involved harvesting the bacterial cells centrifuging them at 6000g for 15 minutes at 4°C to pellet the cells. 10mL of Buffer 1 was used to resuspend the cell pellet. 10mL of Buffer P2 was then added and the tube inverted 6 times to mix and was incubated for 5 minutes at room temperature. Then 10mL of chilled Buffer P3 was added and tube inverted 6 times to mix. The tube was incubated on ice for 20 minutes. Subsequently, the tubes were centrifuged for 20,000g for 30 minutes at 4°C. Meanwhile, 10mL of Buffer QBT was added to QIAGEN-tip to equilibrate it and flow-through was discarded. After the centrifugation, the supernatant was applied to the QIAGEN-tip (provided in kit) and allowed to flow-through by gravity flow. The QIAGEN-tip was washed twice with 30mL of Buffer QC. To elute the DNA, 15mL of Buffer QF was added and collected into a 50mL tube. To precipitate the DNA, 10.5mL (0.7 volumes) of isopropanol was mixed with the eluted DNA and centrifuged at 4025g for 1 hour at 4°C. The supernatant was carefully decanted. The DNA pellet was then washed with 5mL of 70% ethanol and centrifuged at 4025g for 20 minutes at 4°C. The supernatant was carefully pipetted off. The DNA pellet was left to air dry for 10 minutes before resuspending the DNA in 250µL of nuclease-free water. The DNA was stored in -20°C.

2.24.8 Lentiviral particle production

Lentiviral particles were generated using a 3rd generation packaging system approach (Dull et al. 1998). 5.25x10^{6} HEK293Ts were seeded in T75 flasks 18-24 hours prior to transfection. 22.5µg DNA consisting of eGFP-CD63 or mCherry-CD63 plasmids (plasmid

of interest; Appendix 1 and 2), pMDLg/pRRE and pRSV-Rev (packaging plasmids) and pCMV-VSV-G (envelope plasmids) were mixed at a 4: 1.5: 1.5: 1 ratio (w/w/w) in 'DMEM serum-free media'. Tranfection agent, polyethylenimine (PEI; Sigma Aldrich), was added to this mixture at a PEI:DNA ratio of 3:1 (w/w) and incubated for 15 minutes at room temperature. HEK293Ts were visualised under light microscopy to ensure they had adhered to the flask surface and consisted of 70-90% confluency prior to removal of the culture supernatant. Media was replaced with the DNA/DMEM/PEI mixture and 'DMEM complete media' added at a final volume of 8mL before being incubated for 48-56 hours at 37°C in the presence of 5% (v/v) CO_2. Transfection efficiency was visually assessed by fluorescence microscope prior to harvesting the viral particles within the culture media. The supernatant of the HEK293Ts containing the viral particles were harvested and filtered through a 0.45µm filter to remove cell debris. Cold PEG-it™ Virus Precipitation Solution (Systems Biosciences, SBI) were then added to the filtered supernatant at a 1:4 ratio of PEG-it™:supernatant (v/v) and incubated at 4°C for 18 hours. The viral particles were then pelleted by centrifugation at 2500g for 30 minutes at 4°C before resuspension in a 10^{th} of the original PEG-it™:supernatant volume in 'human complete media' and stored at -80°C until usage.

2.24.9 Transduction of human Tregs with lentiviral particles

4.8µg of RetroNectin® (Takara, Kusatsu, Shiga Prefecture, Japan) in PBS was added each well of a non-tissue-culture-treated 24 well plate (Falcon®, Corning) and incubated at 4°C overnight. Each well was washed with PBS prior to adding 50µl of thawed lentiviral particles. $0.5x10^6$ human Tregs (day 3 post-isolated Tregs and stimulated with anti-CD3/CD28 beads) were added per well of RetroNectin®-coated wells and spinoculated at 600g for 1 hour at 30°C, after which cells were incubated at 37° in the presence of 5% (v/v) CO_2. On days 7 and 10 post transduction, the transduction efficiency was tested by flow cytometry assessment of eGFP or mCherry expression on a BD LSRFortessa™ and analysed on FlowJo™.

2.25 Fluorescence-assisted cell (FACS) sorting

On day 10, transduced human Tregs were harvested and activation beads removed by magnetic separation. Cells were washed twice with FACS buffer and resuspended at a maximum concentration of $20x10^6$ cells per 1mL. Cells were gated on high $eGFP^+$ or high $mCherry^+$ expression and purified using a BD FACSAria II (BD Biosciences). Cells were sorted into 'human complete media'.

2.26 Statistical Analysis

For two group comparisons, two-tailed Student *t*-test was used for statistical significance analysis. For three or more group comparisons, One-way ANOVA with Tukey multiple comparison post-hoc test was used to test statistical significance. Data represent mean +/- standard deviation (SD) or +/- standard error of the mean (SEM). Significance is denoted as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= non-significant.

Chapter 3
Characterisation of Mouse Treg EVs

Chapter 3 - Characterisation of Mouse Treg EVs

3.1 Introduction

3.1.1 $CD4^+$ $CD25^+$ Mouse Treg derived EVs

In 2013, my host laboratory was the first group to demonstrate that mouse $CD4^+$ $CD25^+$ Tregs release $CD63^+$ $CD81^+$ EVs upon activation (Lesley Ann Smyth et al. 2013). They showed that these EVs were able to suppress the proliferation of $CD4^+CD25^-$ T cells *in vitro*. Furthermore, these EVs' suppressive capacity was dose dependent with Treg EVs derived from $8x10^7$, $5x10^7$ and $3x10^7$ Tregs providing a 50%, 30% and 25% inhibition of $CD4^+CD25^-$ T cell proliferation, respectively. However, the authors did not state the number of EVs produced per cell. Nonetheless, the pro-inflammatory cytokines that activated $CD4^+CD25^-$ T cells produce, IL-2 and IFNγ, were significantly reduced by the presence of Treg EVs (Lesley Ann Smyth et al. 2013).

3.1.2 EV release is important for Treg function

In 2014, Okoye *et al.* (Okoye et al. 2014) confirmed and expanded the findings of Smyth *et al*. (Lesley Ann Smyth et al. 2013). They showed that mouse $CD4^+$ $CD25^+$ Tregs released exosomes upon their activation, although these were only on the freshly isolated Tregs whereas Smyth *et al.* used both freshly isolated mouse Tregs and mouse Treg cultured lines. Okoye *et al.* also suggested that the release of exosomes was an essential component for Tregs to functionally suppress target cells (Okoye et al. 2014). Rab27α and Rab27β proteins have previously been shown to be involved in docking of multivesicular endosomes to Rab27 effector proteins on the plasma membrane of the cell; demonstrating that Rab27α and Rab27β have important roles in exosome release by various cells, which were experimentally confirmed by various groups (M. Fukuda 2013; Ostrowski et al. 2010; R. K. Singh et al. 2012). $Rab27^{ashen/ashen}Rab27\beta^{-/-}$ double knockout mouse (Rab27αβ DKO) derived from Tregs released less exosomes than control Tregs. Furthermore, Rab27αβ proteins are required for Treg-mediated suppression given that Rab27αβ DKO Tregs did not prevent T cell proliferation. Additionally, the Rab27αβ DKO Tregs were unable to prevent inflammatory disease in mice with visible signs of disease progression including shortening of the colon, weight

loss and increased IFNγ expression levels compared to control mice. Hence, this highlights the importance of Rab27αβ-dependent release of exosomes for Treg functions *in vitro* and *in vivo* (Okoye et al. 2014). Rab27αβ DKO Tregs were impaired in their ability to transport dsRNA into conventional T effector cells, deducing that an exosomal-release-related pathway plays a principal role for transferring RNA into target T cells. Interestingly, the Rab27αβ DKO Tregs had a similar phenotype, transcriptional profile and tetraspanin expression levels to wild type Tregs despite not being able to suppress Th1 cell proliferation, suggesting that exosome release from Tregs is a major contributor to Treg-mediated suppression *in vitro* (Okoye et al. 2014).

3.1.3 Regulated release of EVs from Tregs

Activated Tregs released more $CD63^+$ exosomes per cell compared to activated or stimulated naïve $CD4^+$ cells; naïve $CD8^+$ cells; B cells; Th17 cells or Th1 cells (Okoye et al. 2014). Tregs are known to be regulated by various compounds including IL-2 (Fontenot et al. 2005; Boyman et al. 2006), amphiregulin (Areg) (Zaiss et al. 2013) and ATRA (Nolting et al. 2009), these compounds were also shown to regulate the release of mouse Treg exosomes (Okoye et al. 2014). Treg release of exosomes were influenced by changes in intracellular calcium, hypoxic conditions and the biosynthesis of ceramide (Okoye et al. 2014), similar to the findings by other groups showing that these factors also regulate exosome release from other cell types (Savina et al. 2003; H. W. King, Michael, and Gleadle 2012; Trajkovic et al. 2008).

3.1.4 Suppressive molecules expressed by Treg EVs

Smyth *et al.* showed that Treg EVs displayed CD73, CD25 and CTLA-4 molecules on their surfaces which are molecules also found on the parent cell, these molecules were shown previously to be involved in different mechanisms of immunosuppression (see section 1.6.3). Albeit CTLA-4 presence on these Treg EVs, this was redundant as the authors demonstrated that blocking CTLA-4 did not affect their suppressive capabilities. In addition, although CD25 molecules were found on Treg EVs, it was also found on the control $CD4^+$ T cell EVs. Given that control $CD4^+$ T cell EVs were not suppressive but

instead pro-inflammatory the authors suggested that CD25 was not involved in immunosuppression; however, this was not clarified experimentally by the authors.

Instead, the authors demonstrated that the presence of CD73 on Treg EVs contributed to the immunosuppression observed. CD39 is a cell surface-located enzyme that catalyses pro-inflammatory ATP into AMP. AMP is then taken up by the ectoenzyme CD73 that converts AMP to anti-inflammatory adenosine. Adenosine is taken up by adenosine receptors $A_{2a}R$ which are found on activated Teffs (Chhabra et al. 2012; L. Han et al. 2017; Leone et al. 2018), this initiates intracellular cAMP production resulting in decreased cytokine production and thus preventing T cell pro-inflammatory responses (Romio et al. 2011). Given that adenosine was detected in the co-cultures of $CD73^+$ Treg EVs with exogenous 5'AMP, it firstly confirmed that the CD73 molecules present on Treg EVs were functional in converting 5'AMP into adenosine. Secondly, from the results it is plausible that the release of EVs bearing CD73 on their surfaces increased the surface area in which this ectoenzyme could function within the local microenvironment, and thus ultimately promoting the various ways in which Tregs can suppress and function. Smyth *el al.* suggested that this occurrence could explain the observed inhibition of T cell responses (Lesley Ann Smyth et al. 2013). However, when the authors inhibited CD73 activity on EVs through the use of a CD73 chemical inhibitor APCP, this accounted for most of the immunosuppression observed, although some level of immunosuppression was unaccounted for, suggesting that other factors or molecules may be involved in driving Treg EVs to suppress T cell proliferation and pro-inflammatory cytokine production. Given that EVs can act like vehicles to deliver not only cell surface molecules but also other types of biological material such as RNA, proteins, cytokines and DNA into target cells (Clotilde Théry, Zitvogel, and Amigorena 2002; Colombo, Raposo, and Théry 2014; McKelvey et al. 2015; Graça Raposo and Stoorvogel 2013), it is conceivable that mouse Treg EVs could be carrying more than just CD73 to promote inhibition of T cell responses.

3.1.5 miRNA contained within EVs

Importantly, mouse Treg exosomes contained pre-mature miRNAs, mature miRNAs and mRNAs (Okoye et al. 2014), similar to the findings by other groups showing T cells

releasing RNA-containing exosomes (Mittelbrunn et al. 2011; Ventimiglia and Alonso 2016; X. Wang et al. 2018). The Treg exosomal miRNA repertoire were different compared to the parent cells, or exosomes isolated from Th1 or Th2 cells (Okoye et al. 2014), indicative of potential diverse roles for Treg exosomes. In particular, Let-7d contained in Treg exosomes were found to contribute to their suppressive ability by inhibiting Th1 cell proliferation and IFNγ production. Let-7d was demonstrated to be a major contributor for Treg exosomes-mediated immunosuppression both *in vitro* and *in vivo*. Additionally, miRNA transcripts present in Treg exosomes were linked to molecular pathways that suppress T cells, suggesting that miRNA molecules could play a role in inhibition of protein synthesis or regulation of gene expression in the target T cells leading to an anti-inflammatory outcome (Okoye et al. 2014).

However, to test this possibility, miRNA deficient cells and exosomes were required. A commonly used miRNA deficient system is Dicer (Lewis and Steel 2010; Krill et al. 2013; Bernstein et al. 2003; Kanellopoulou et al. 2005; Park, Choi, and McManus 2010). Dicer is an enzyme required to cleave double stranded premature miRNA into functional mature miRNA strands (Lewis and Steel 2010; Bernstein et al. 2003; Kanellopoulou et al. 2005; Park, Choi, and McManus 2010; Krill et al. 2013). Okoye *et al*. demonstrated that the $Dicer^{-/-}$ Treg exosomes were unable to modulate Th1 cell proliferation and IFNγ cytokine production like their wild-type Treg exosomes counterparts. $Dicer^{-/-}$ T cells received miRNAs from Tregs when co-cultured together suggesting that miRNA in Tregs is transferred between cells (Okoye et al. 2014).

Recently, work performed by another member of the host laboratory investigated the miRNA expression profile in Tregs and DCs. The intended aim of the miRNA screen was to investigate which miRNAs are highly expressed in murine Treg lines and hence most probable to be transferred into DCs which alter their function and phenotype in such a way to inhibit DCs from priming T cells. The results revealed differential expression levels of various miRNAs including miR-142-3p and miR-150-5p. Notably, miR-150 expression levels were significantly higher in Tregs compared with DCs. These results are linked to the data generated and presented in this chapter. Namely, this chapter aimed to investigate whether these Tregs transferred miRNA via their EVs and thus formed the majority of this results chapter.

3.2 Aims and objectives

Aim

As described in section 1.6.3, Tregs regulate the immune responses by acting on T cells and DCs. Okoye *et al.* demonstrated that mouse Treg exosomes can transfer miRNA into T cells to affect their function (Okoye et al. 2014), herein I investigated whether mouse Treg EVs transfer miRNAs to BM-DCs and in doing so modulate their phenotype and function.

Objectives:

(1) Investigate which miRNAs are associated with mouse Treg EVs, and of these, which were higher expressed compared to mouse FoxP3^{low} T cell EVs.
(2) Test if these mouse Treg and FoxP3^{low} T cell EVs are acquired by BM-DCs.
(3) Investigate whether mouse Treg EVs alter DC function by affecting co-stimulatory molecule expression and cytokine production by BM-DCs.

3.3 Results

3.3.1 BALB/c BM-DCs phenotype

BALB/c BM-DCs were generated from BALB/c mice bone marrow precursor cells cultured in the presence of GM-CSF, for 6 days, and harvested at the end of culture for phenotypic analysis by flow cytometry. The average percentage CD11c BM-DCs that were present in the cultures was 89.6±2.7% suggesting the presence of DCs. These cells also expressed CD80 (90.1±5.7%), CD86 (73.1±19.7%), H2K^{d} (MHC class I; 98.7±1.6%) and of IA^{d} (MHC class II; 79.5±14.0%) (Figures 3.1-A-C), which are cell surface markers commonly found present on DCs and frequently used to identify them (Shortman and Liu 2002; Pulendran et al. 1997; Hua et al. 2012).

3.3.2 Mouse Tregs and T cell phenotype

In this chapter, two mouse cell lines with direct allospecificity (generated by co-culture with BALB/c BM-DCs) were used. Both cell lines expressed CD4, CD25 and one of the T

cells were FoxP3^{high} (Tregs) and the other expressed low levels of FoxP3 (FoxP3^{low} T cell line). The background of the genetic information and the nomenclature of the strain of origin are presented in **Table 3.1**. A phenotypical analysis of Tregs and FoxP3^{low} T cell lines generated demonstrated that 95.1±4.7% of Tregs expressed CD4^{+}, of these 87.5±9.2% expressed CD25^{+}, and were FoxP3^{high} and CD73^{+} (**Figure 3.2-A**). Whereas, 97.0±4.8% of (control cells) T cells were CD4^{+} and 90.2±9.6% of these were CD25^{+} as well as FoxP3^{low} and CD73^{low} (**Figure 3.2-B**). In mouse, FoxP3 is a lineage-defining Treg transcription factor (S Sakaguchi et al. 1995), a high percentage of Tregs expressed this marker (80.6±10.1%) compared to the FoxP3^{low} T cells (27.9±24.1%) (**Figures 3.2-A-C**). The percentage of Tregs expressing ectoenzyme CD73 were notably different between Tregs (98.9±0.6%) and FoxP3^{low} T cells (42.2±8.6%) (**Figures 3.2-A-C**), as previously shown (Lesley Ann Smyth et al. 2013).

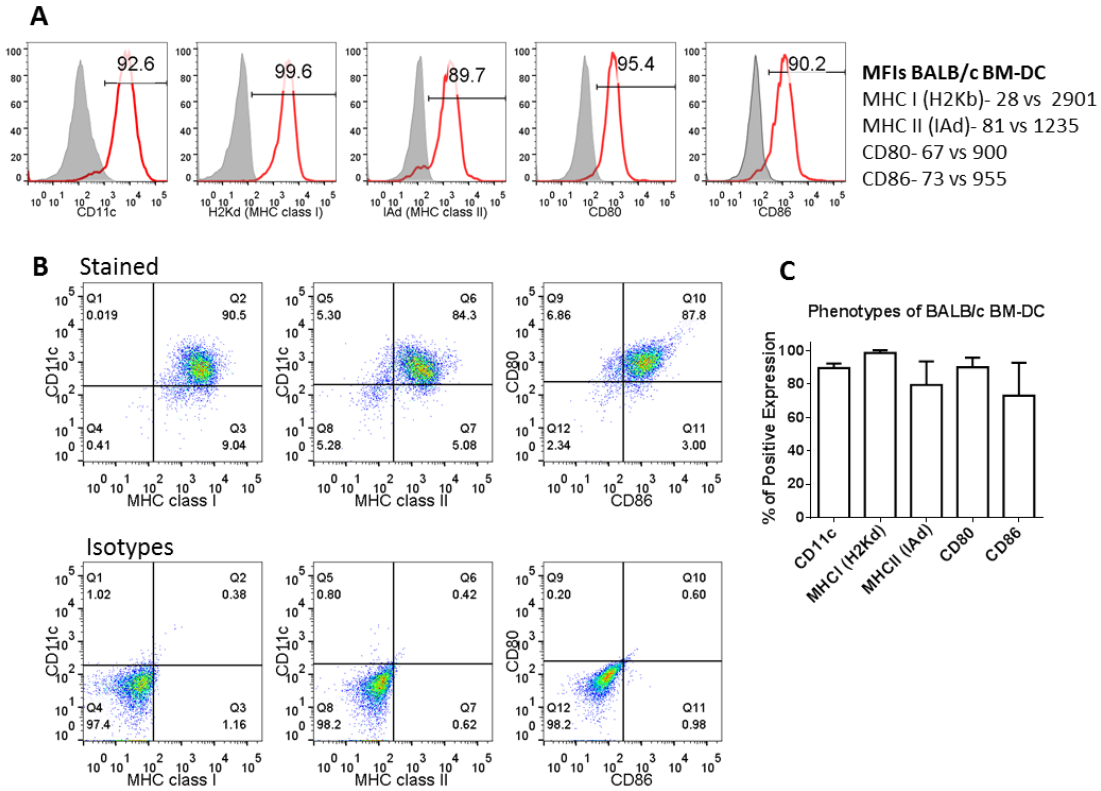


Figure 3.1 Phenotype of BALB/c BM-DCs

(A) *Flow cytometry histograms showing the phenotype of BM-DCs following immunostaining cells with fluorescently-conjugated antibodies anti-mouse specific for CD11c, MHC class I (H2K^{b}), MHC class II (IA^{d}), CD80 and CD86 (red lines). Gate positions*

were defined on cells that were immunostained with respective isotype controls (grey solid line). The percentage of immunostained cells within the gate are indicated within each plot. Mean fluorescent intensity values (MFIs) are indicated for isotype versus stained cells. The y-axis indicates the percentage of maximum expression. ***(B)*** *Flow cytometry dot plots of the phenotype of BM-DCs. Gate positions were defined on cells that were immunostained with respective isotype controls. The percentage of immunostained cells within the quadrant gates are indicated within each plot.* ***(C)*** *Pooled data of BM-DC phenotype. Data shown is mean+SD from 4 individual experiments.*

Cell line	**Mouse strain cell are derived from**	**Stimulant**	**Description**
Direct Tregs and FoxP3^{low} T cell	C57BL/6	K^{d} antigen from BALB/c BM-DCs	CD4^{+} CD25^{+} Tregs/T cells isolated C57BL/6 mice. Tregs/T cells were cultured with BALB/c BM-DCs on a weekly basis at a 4:1 ratio of Tregs/T cells:DCs, to generate 'antigen-specific direct Tregs/T cells'
Rab27αβ DKO Tregs	Rab27α$^{ashen/ashen}$ β$^{-/-}$ knock-out	K^{d} antigen from BALB/c BM-DCs	CD4^{+} CD25^{+} Tregs isolated from Rab27α$^{ashen/ashen}$ β$^{-/-}$ knock-out mice. Tregs were cultured with BALB/c BM-DCs on a weekly basis at a 4:1 ratio of Tregs:DCs, to generate 'antigen-specific direct EV-deficient Tregs'
BALB/c BM-DCs	BALB/c	GM-CSF	BM-DCs progenitors were cultured in the presence of GM-CSF.
Dicer$^{-/-}$ BM-DCs	Rosa26-ERT2-Cre Dicer floxed/floxed	GM-CSF +/- 4OHT (Tamoxifen) or ethanol (vehicle alone)	BM-DCs progenitors were cultured in the presence of GM-CSF with or without 4OHT (Tamoxifen) or 100% ethanol (vehicle).

Table 3.1- Mouse cell lines generated

Mouse cell lines with genetic background

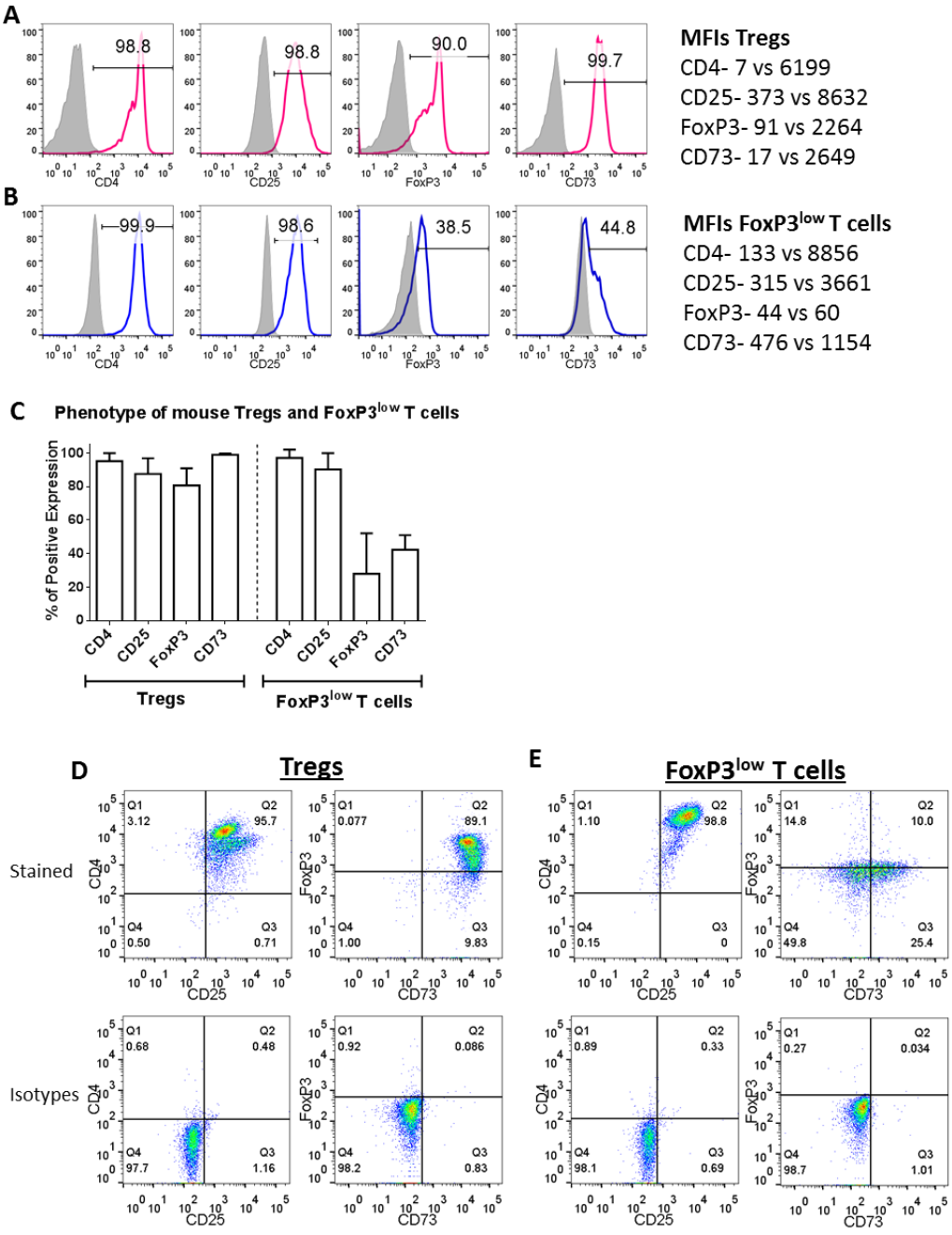


Figure 3.2- Phenotype of mouse Tregs and FoxP3^{low} T cells

Flow cytometry histograms showing the phenotype of ***(A)*** *Tregs and* ***(B)*** *FoxP3^{low} T cells as determined by immunostaining cells with fluorescently-conjugated antibodies anti-mouse specific for CD4, CD25, FoxP3 and CD73. Gate positions were defined on cells that were immunostained with respective isotype controls (grey solid line). The percentage of immunostained cells within the gate are indicated within each plot. Mean fluorescent intensity values (MFIs) are indicated for isotype versus stained cells. The y-axis indicates the percentage of maximum expression.* ***(C)*** *Pooled data of Tregs and FoxP3^{low} T cell*

phenotype. Data shown is mean+SD from 6 individual experiments. Flow cytometry dot plots showing the phenotype of ***(D)*** *Tregs and* ***(E)*** *FoxP3^{low} T cells specific for CD4, CD25, FoxP3 and CD73. Gate positions were defined on cells that were immunostained with respective isotype controls. The percentage of immunostained cells within the quadrant gates are indicated within each plot.*

3.3.3 Generation and phenotype of Rab27αβ DKO Treg line

As previously mentioned, Rab27αβ proteins are important for EV release from cells (Tolmachova et al. 2007; Okoye et al. 2014). To test the importance of EVs in Treg function, a Rab27αβ DKO mouse Treg line from Rab27αβ DKO mice (a kind gift from Dr Tanya Tolmachova of Imperial College London, UK) were generated. The Rab27αβ DKO Tregs were isolated from the Rab27αβ DKO mice spleen and lymph nodes using a negative selection for $CD4^+$ cells and then a subsequent $CD25^+$ positive selection. These Rab27αβ DKO Tregs were stimulated with BALB/c BM-DCs to generate BALB/c allo-antigen specific Rab27αβ DKO Tregs. A phenotypical analysis of Rab27αβ DKO Tregs demonstrated that 82.4% of Tregs expressed $CD4^+$, of these; 69.0% expressed $CD25^+$; 83.6% expressed $FoxP3^+$ and 91.1% expressed $CD73^+$ (Figures 3.3-A-C). Thus, Rab27αβ DKO Tregs expressed common Treg markers.

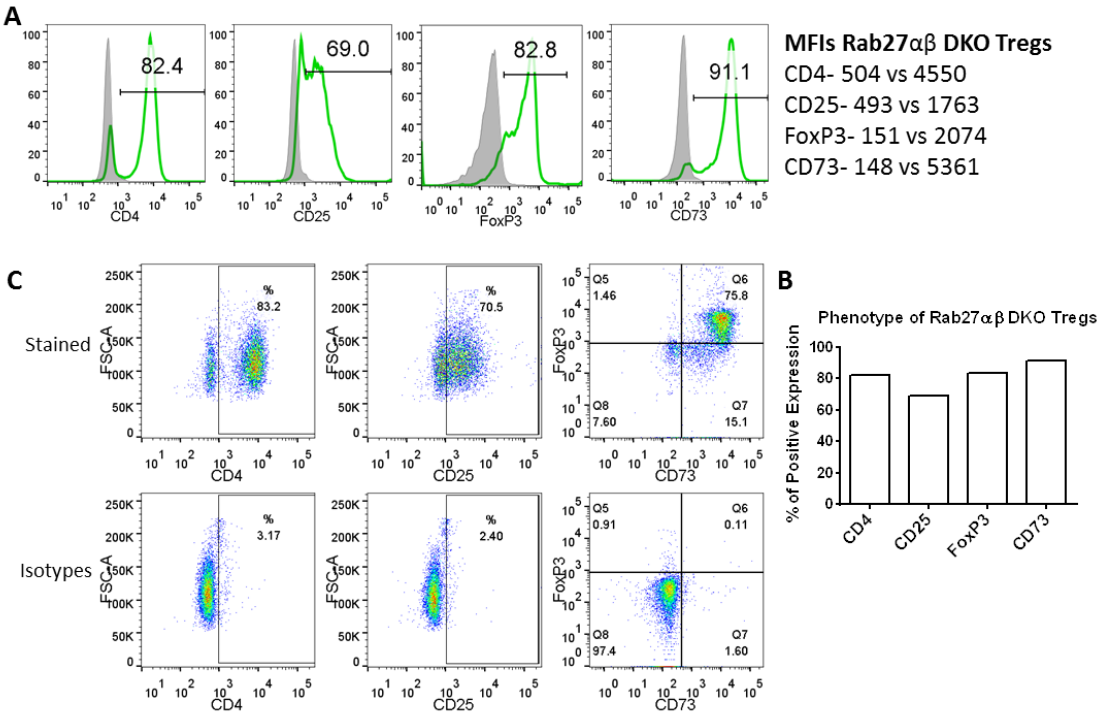


Figure 3.3- Phenotype of Rab27αβ DKO Tregs

(A) *The phenotype of Rab27αβ DKO Tregs was determined by immunostaining cells with fluorescently-conjugated antibodies anti-mouse specific for CD4, CD25, FoxP3 and CD73 (green lines). Gate positions were defined on cells that were immunostained with respective isotype controls (grey solid line). The percentage of immunostained cells within the gate are indicated within each plot. Mean fluorescent intensity values (MFIs) are indicated for isotype versus stained cells. The y-axis indicates the percentage of maximum expression.* ***(B)*** *Bar graph of Rab27αβ DKO Tregs phenotype. Data represents one experiment.* ***(C)*** *Flow cytometry dot plots showing the phenotype of Rab27αβ DKO Tregs specific for CD4, CD25, FoxP3 and CD73 as determined by immunostaining. Gate positions were defined on cells that were immunostained with respective isotype controls. The percentage of immunostained cells within the gates are indicated within each plot.*

3.3.4 Tregs, FoxP3^{low} T cells and Rab27αβ DKO Tregs all inhibit T responder cells (Tresps) proliferation

After confirming that the Treg lines and FoxP3^{low} T cells expressed markers associated with Tregs and T cells, respectively, it was important to test whether these cells suppressed T cells. To accomplish this, suppression assays were set up as described in section 2.4. To test whether the Tregs suppressed alloreactive Tresps, a thymidine-based suppression assay was used. Compared to the co-culture wells consisting of B6 CD4$^+$ Tresps with BALB/c APCs, the proliferation was significantly reduced in the presence of equal numbers (to Tresps) of Tregs (Figure 3.4-A). To further confirm that the Tregs were suppressive, a polyclonal suppression assay was performed using B6 CD4$^+$ Tresps, which were labelled with the proliferation dye CFDA-CFSE. These cells were then co-cultured with unstained BALB/c APCs and αCD3 antibody and the Tregs or FoxP3^{low} T cells. By doing this, the responses of the B6 CD4$^+$ Tresps were specifically detected compared to ^{3}H-thymidine labelling, which is incorporated in all the dividing cells in the co-culture well. After 3 days of co-culture, the cells were harvested and acquired by flow cytometry to measure CFSE dilution.

Tregs suppressed the CD4$^+$ Tresps proliferation induced by APCs and αCD3 antibody and thus suppressed in a polyclonal manner (Figures 3.4-B and C), confirming the results obtained by using ^{3}H-thymidine-based suppression assays (Figure 3.4-A). A similar suppression albeit to a lower level was observed with the FoxP3^{low} T cell line, thus the FoxP3^{low} T cell line was suppressive but not as potently suppressive as the Tregs (Figure 3.4-D). Despite this observation, the FoxP3^{low} T cell lines, because of its low expression of FoxP3 and CD73 was used as a control for future work characterising EVs produced by the Tregs.

As mentioned in section 3.1.2, Okoye *et al.* demonstrated that Rab27αβ DKO Tregs were not able to suppress the proliferation of T cells, thus indicating the importance of EVs for Treg suppressive ability (Okoye et al. 2014). Tregs with direct allospecificity (by culturing with BALB/c BM-DCs) was generated from the Rab27αβ DKO mice. In contrast to the results from Okoye *et al.* (Okoye et al. 2014) the Rab27αβ DKO Tregs were suppressive (Figure 3.4-E). However, the Rab27αβ DKO Tregs were difficult to expand in

numbers and also there were particles/EVs that were detected from the culture supernatant of these cells (data not shown). The Rab27αβ DKO Tregs were not fully characterised in terms of vesicle deficiency due to limited access to the Rab27αβ DKO mice. Consequently, the Rab27αβ DKO Tregs were not further used in analyses for this PhD project. Instead, only the FoxP3^{low} T cell line was used as a control cell line to compare with the Tregs.

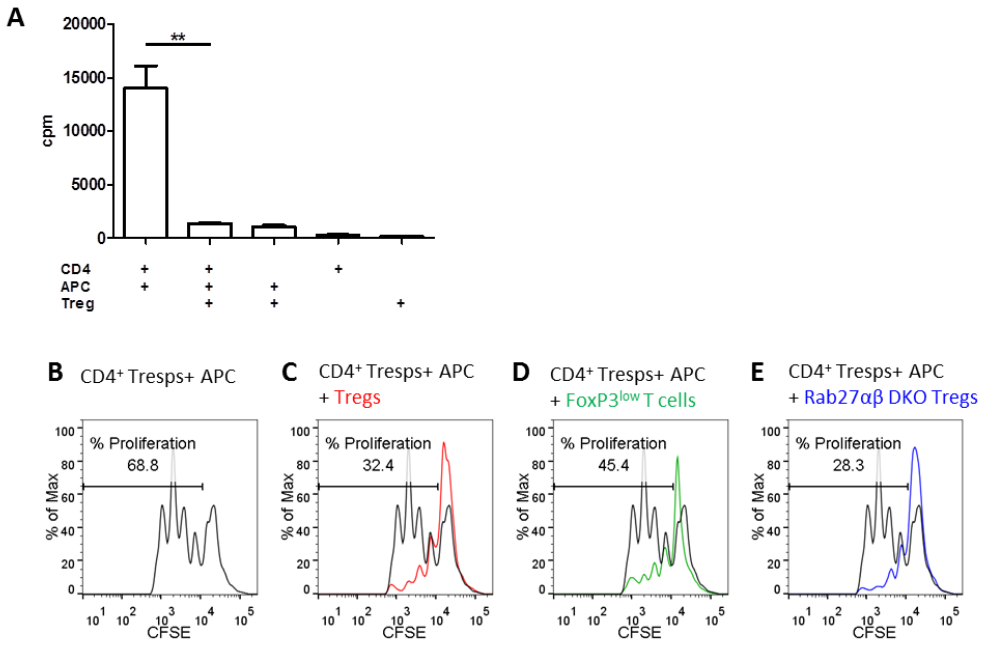


Figure 3.4- Tregs, FoxP3^{low} T cells and Rab27αβ DKO Tregs inhibit Tresps proliferation

(A) *Alloantigen specific suppressive capacity of Tregs as tested by thymidine-based suppression assays. B6 CD4^{+} Tresps were stimulated with BALB/c APCs in the presence or absence of an equal number of Tregs. Co-cultures were performed for 3 days and proliferation was measured by ^{3}H-thymidine incorporation. Data are representative of 5 experiments.* ***(B to E)*** *B6 CD4^{+} T cells were stimulated with BALB/c APCs, αCD3 antibody and in the* ***(B)*** *absence or presence of an equal number of* ***(C)*** *Tregs,* ***(D)*** *FoxP3^{low} T cells or* ***(E)*** *Rab27αβ DKO Tregs. Polyclonal suppressive capacity of Tregs, FoxP3^{low} T cells and Rab27αβ DKO Tregs was tested by CFSE-based suppression assays. Co-cultures were performed for 3 days and proliferation was measured by CFSE dilution. Percentage of proliferation gate was set from CFSE-stained unstimulated B6 CD4^{+} T cells. Data are representative of 3 independent experiments.*

3.3.5 Mouse Tregs release EVs upon activation

As mentioned above, mouse Tregs release exosomes upon activation as previously shown by Smyth *et al*. (Lesley Ann Smyth et al. 2013) and Okoye *et al.* (Okoye et al. 2014). To confirm that the BALB/c allospecific Tregs studied in this project also released EVs, Tregs were activated using plate-bound αCD3/CD28 antibodies and 24 hours later EVs were isolated by ultracentrifugation. Isolated particles were examined under

electron microscopy (EM) and NanoSight analysis with Nanoparticle Tracking Analysis (NTA) software. Under the EM, the particles had a morphology that was 'cup-shaped' and approximately 100nm in diameter (**Figure 3.5-A**), as previously reported for exosomes (Colombo, Raposo, and Théry 2014; Clotilde Théry, Zitvogel, and Amigorena 2002; Akers et al. 2013; Graça Raposo and Stoorvogel 2013). As processing EVs for EM analysis can modify the size of particles (Y. Wu et al. 2015), NanoSight was also used to confirm the particle sizes. Using NanoSight it was demonstrated that these particles had an average mean of 117.8 ± 4.6nm and an average mode of 82.0 ± 4.7nm (**Figures 3.5-B and C**), confirming the EM results (**Figure 3.5-A**). Due to the limited access to both EM and NanoSight equipment, the FoxP3^{low} T cells EVs were not assessed by either modalities. Taken together, mouse Tregs released EVs upon activation and these EVs were cup-shaped and approximately 100nm in diameter.

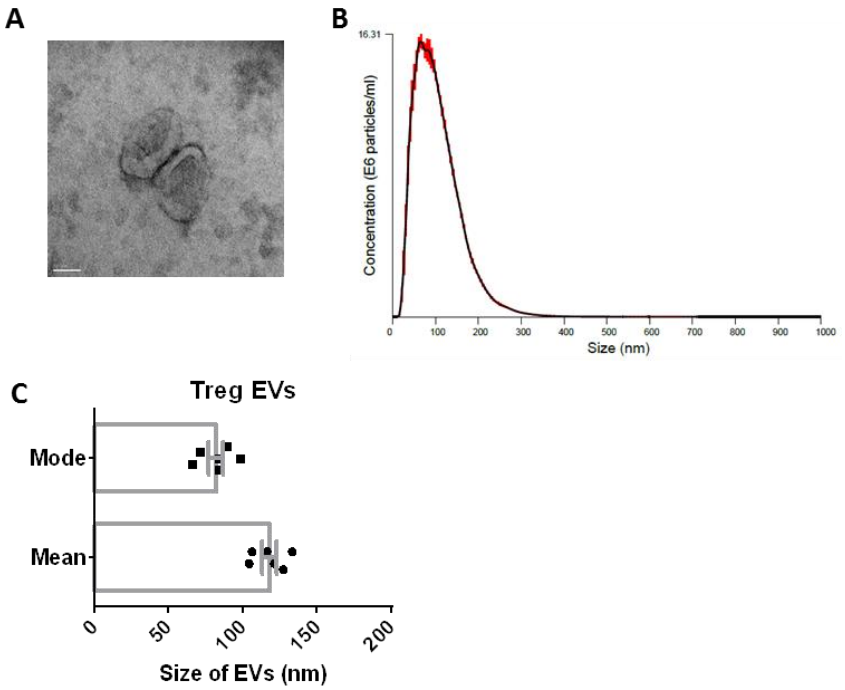


Figure 3.5- Mouse Tregs release EVs upon activation

***(A)** Transmission electron microscopy image of EVs released from Tregs activated by plate-bound αCD3/CD28 antibodies following ultracentrifugation isolation. Scale bar indicates 50nm. A representative transmission electron microscopy image from 3 independent experiments is shown. **(B)** Particle size distribution of Treg EVs isolated from culture supernatants by ExoQuick-TC™ following overnight activation with plate bound*

αCD3/CD28 antibodies. Particles were measured on a NanoSight LM-10 instrument. Representative histogram showing the average EV sizes and their concentration, measured from 3 readings. Black lines show the mean value with red lines showing ± SD. Histogram is representative of 6 independent experiments. ***(C)*** *Pooled data of NanoSight readings of Treg EVs mode and mean sizes of particles. N=6 individual samples. Each dot represents the average reading derived from 3 video NTA NanoSight measurements in each of the 6 individual samples. Bars show mean± standard deviation.*

3.3.6 Treg EVs and FoxP3^{low} T cell EVs are acquired by BM-DCs

Okoye *et al.* have previously shown that T cells can acquire Treg exosomes *in vitro* (Okoye et al. 2014). As mentioned in **section 1.6.3**, DCs represent key target cells of Tregs, therefore, to investigate whether DCs can acquire Treg EVs the following experiment was performed. CFSE-labelled Tregs or FoxP3^{low} T cells (**Figures 3.6-A and B**, respectively) were activated with plate-bound αCD3/CD28 antibodies. To test that Tregs and FoxP3^{low} T cells had been activated, the upregulation of CD69, an activation marker of T cells (Ziegler, Ramsdell, and Alderson 1994; Simms and Ellis 1996) was assessed (**Figures 3.6-C and D**, respectively). After confirmation of their activation state, the CFSE^{+} Treg EVs or CFSE^{+} FoxP3^{low} T cell EVs were isolated from the supernatant and co-cultured with BM-DCs for 24 hours. BM-DCs were washed extensively to remove unbound or unincorporated CFSE^{+} EVs. The presence of CFSE in BM-DCs were then evaluated by flow cytometry. Flow cytometric analysis showed that BM-DCs had acquired the CFSE^{+} Treg EVs and CFSE^{+} FoxP3^{low} T cell EVs as their MFI had increased from 81 (BM-DCs alone) to 108 (BM-DCs co-cultured with CFSE^{+} Treg EVs) and 131 (BM-DCs co-cultured with CFSE^{+} FoxP3^{low} T cell EVs) (**Figures 3.6-E and F**, respectively), suggesting that CFSE^{+} Treg EVs and CFSE^{+} FoxP3^{low} T cell EVs had been acquired by BM-DCs.

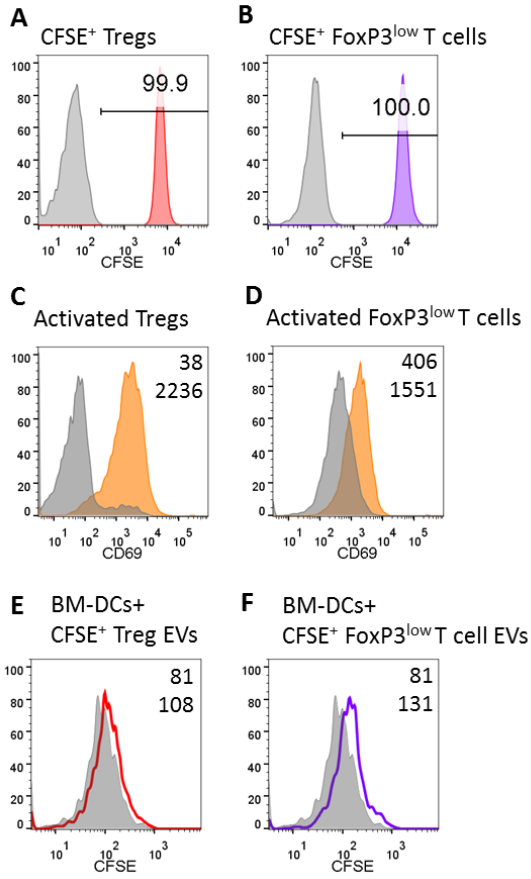


Figure 3.6- Treg EVs and FoxP3^{low} T cell EVs are acquired by BM-DCs

(A) *Tregs or* ***(B)*** *FoxP3^{low} T cells were labelled with CDFA-CFSE. Activation of* ***(C)*** *Tregs or* ***(D)*** *FoxP3^{low} T cells was performed using plate bound αCD3/CD28 antibodies and 24 hours later, assessed by testing the upregulation of CD69 expression using flow cytometry. Grey solid line shows CD69 expression in unstimulated Tregs or FoxP3^{low} T cells whereas orange shows the αCD3/CD28 activated cells. Supernatant was subsequently collected, cells and cell debris depleted and EVs isolated via ultracentrifugation.* ***(E and F)*** *BM-DCs were cultured alone (grey) or co-cultured with CFSE^{+} Treg EVs or CFSE^{+} FoxP3^{low} T cell EVs for 24 hours. Flow cytometry histogram plots showing CFSE expression levels of BM-DCs cultured alone (grey) and BM-DCs co-cultured with* ***(E)*** *(red) CFSE^{+} Treg EVs or* ***(F)*** *(purple) CFSE^{+} FoxP3^{low} T cell EVs. Mean fluorescent intensity values are indicated for isotype (top) versus stained cells (bottom). Data is representative of 3 independent experiments. The y-axis indicates the percentage of maximum expression.*

3.3.7 Mouse Treg EVs and FoxP3^{low} T cell EVs are enriched in small RNA species

Previous work has demonstrated that mouse Treg exosomes carry both mRNAs and miRNAs (Okoye et al. 2014). Furthermore, the same authors have shown that both types of RNA species are differentially expressed compared to their parent cells or Th1 cell-derived exosomes (Okoye et al. 2014). To confirm firstly the presence of RNA in the EVs generated in this PhD project, and secondly that Tregs, Treg EVs or FoxP3^{low} T cell EVs contained different types of RNA species, total RNA was isolated from the aforementioned cells and EVs or FoxP3^{low} T cell EVs. The Agilent 2100 Bioanalyser was used to assess the RNA profiles and the results demonstrated that the Treg cellular RNA profile was different compared to both Treg EV RNA and FoxP3^{low} T cell EV RNA. Treg cellular RNA contained the expected 18S and 28S RNA peaks (**Figure 3.7-A**), whereas the Treg EV RNA and FoxP3^{low} T cell EV RNA did not contain 18S nor 28S RNA peaks (**Figures 3.7-B and C**, respectively), in line with previous reports (Enderle et al. 2015; Lässer, Eldh, and Lötvall 2012; Eldh et al. 2012). Furthermore, these Treg EVs were enriched in small RNA molecules compared to the Tregs as seen by their higher fluorescent units (FU) levels at 170-200nt region (**Figures 3.7-B and A**).

The method of EV isolation can influence or affect the type or amount of RNA recovery (Eldh et al. 2012). To test whether the gold standard ultracentrifugation or the ExoQuick-TC™ method of EV isolation differs in the recovery of RNA profile, both of these methods were compared using the same starting material. The Agilent 2100 Bioanalyser results showed that the ExoQuick-TC™ method of EV isolation provided a higher amount of RNA recovery compared to ultracentrifugation method of EV isolation (**Figures 3.7-D and E**), in line with previously published reports (Rekker et al. 2014; Alvarez et al. 2012) and both methods of EV isolation recovered small RNA species.

In summary, mouse Treg EVs are enriched in small RNAs and lack ribosomal RNA. Additionally, EV isolation via ExoQuick-TC™ provides a higher amount of RNA recovery compared to ultracentrifugation.

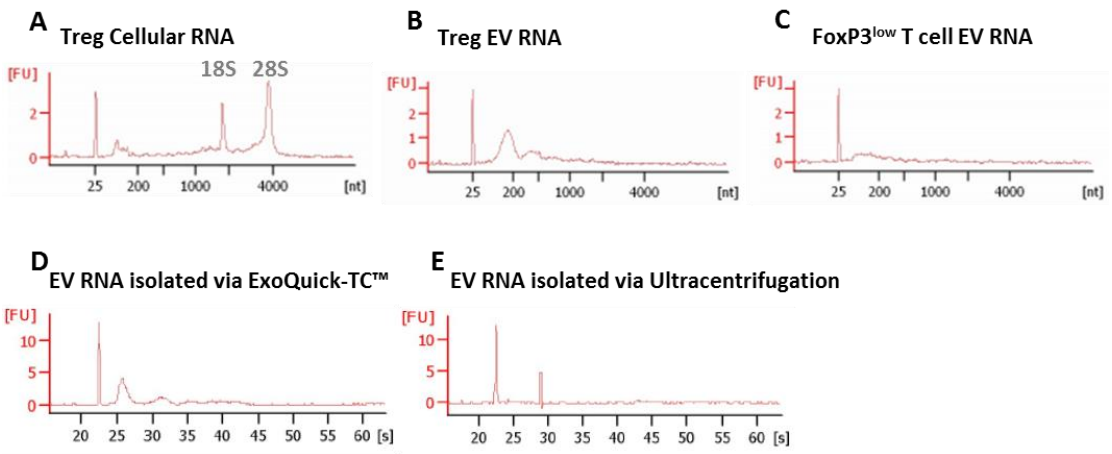


Figure 3.7- Treg EVs and FoxP3^{low} T cell EVs are enriched in small RNA species

Agilent 2100 Bioanalyser analysis of RNA isolated from ***(A)*** *Tregs,* ***(B)*** *Treg EVs or* ***(C)*** *FoxP3^{low} T cell EVs on a total RNA Nanochip. Ribosomal RNA (rRNA) peaks are indicated as 18S and 28S subunits. The data is representative of 2 independent experiments. FU= fluorescence units and nt= nucleotides.* ***(D and E)*** *Two methods of EV isolation were tested in parallel to assess RNA recovery. Treg EVs was isolated using* ***(D)*** *ExoQuick-TC™ or* ***(E)*** *ultracentrifugation and then the same RNA isolation procedure was followed and RNA tested on the Agilent 2100 Bioanalyser.*

3.3.8 Mouse Treg EVs contain miRNAs that are differentially expressed compared to FoxP3^{low} T cell EVs

Okoye *et al.* have shown that mouse Treg exosomes from freshly isolated Tregs contain differential miRNAs compared with Th1 exosomes (Okoye et al. 2014). To investigate whether Treg EVs isolated from my Treg lines contain miRNAs that are differentially expressed compared to FoxP3^{low} T cell EVs, a miRNOME was performed to detect for the presence and expression levels of 754 different miRNAs.

EV miRNAs were profiled using a QuantStudio™ 12K Flex Real-Time PCR system. A volcano plot (**Figure 3.8-A**) was produced to select miRNAs characterized by >1 or <−1 difference between Treg EV and FoxP3^{low} T cell EV content (Log_2 (Fold Change) >1 or <−1) with a $\log_{10}$(p-value)>1.301. Comparing the miRNA profiles of EVs derived from Tregs and FoxP3^{low} T cells, it was observed that many miRNAs were common in

both EVs (**Figure 3.8-A** and **Table 3.2**). However, several miRNAs were differentially detected including three identified miRNAs, miR-142-3p, miR-150-5p and miR-384-5p (**Figures 3.8-B-D**). This was further confirmed by individual qPCR assays. For example, miR-384-5p was present at significantly higher levels in FoxP3^{low} T cell EVs (p=0.0073) (**Figure 3.8-D**), whilst miR-142-3p was present at significantly higher levels in Treg EVs (p=0.0319) (**Figure 3.8-B**). Interestingly, miR-150-5p, a miRNA shown recently to be present in EVs isolated from human CD4^{+} Tregs (Torri et al. 2017), was also found in murine Treg EVs and had significantly higher expression level compared to the FoxP3^{low} T cell EVs (p=0.0381) (**Figure 3.8-C**).

Notably, although the volcano plot showed that miR-150 expression was not significantly different between Treg EVs and FoxP3^{low} T cell EVs (**Figure 3.8-A**), from the individual qPCR assays the miR-150-5p expression was significantly higher in Treg EVs compared to FoxP3^{low} T cell EVs (p=0.0381; **Figure 3.8-C**), highlighting that individual qPCR assays are more sensitive than whole miRNA arrays (Git et al. 2010; Y. Chen et al. 2009), possibly due to difference of normalisation tool used (Pabinger et al. 2014; Ling and Salvaterra 2011; Schwarzenbach et al. 2015). The best normalisation method for a large number of qPCR assays such as a miRNOME is a global mean normalisation which is a value above the mean expression value of all sample cohorts (Mestdagh et al. 2009). Whereas, in individual qPCR assays, due to the small number of samples, a reference house-keeping gene or a reference miRNA which is expressed in similar levels is suggested (Mestdagh et al. 2009). snoRNA are small nucleolar RNAs, that are non-coding but aid chemical modification of other RNAs such as ribosomal RNA, transfer RNA and small nuclear RNA (Scott and Ono 2011). snoRNA202, is generally used as a normalisation candidate (Brattelid et al. 2011), why snoRNA202 presented as enriched in Treg EVs compared to FoxP3^{low} T cell EVs (**Figure 3.8-A**) is unclear, but due snoRNA202 being generally used as a reference gene, it suggested that it might be not involved in Treg EV-mediated actions and thus I did not further validate the expression levels of snoRNA202.

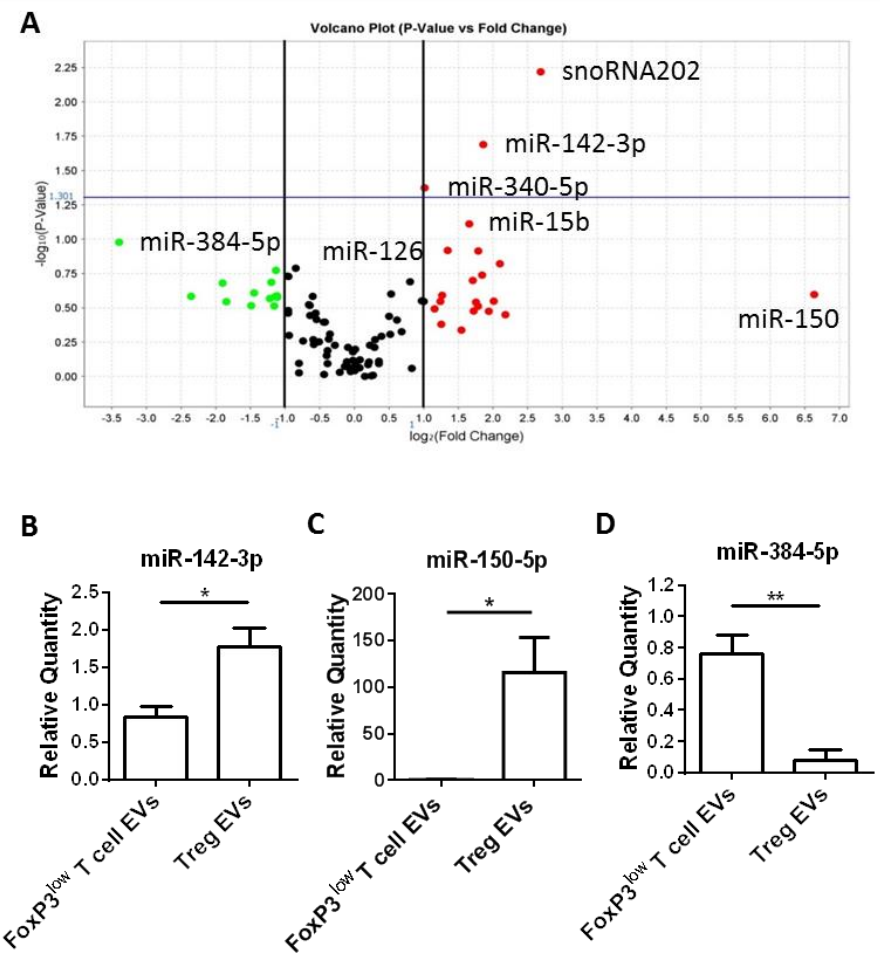


Figure 3.8- Mouse Treg EVs contain miRNAs that are differentially expressed compared to FoxP3^{low} T cell EVs

Mouse Treg EVs and FoxP3^{low} T cell EVs isolated by ExoQuick-TC™ were lysed and total RNA was purified and assessed using NanoDrop™ spectrophotometer and Agilent 2100 Bioanalyser. EVs miRNA was reverse transcribed into cDNA and pre-amplified before miRNA detection using QuantStudio™ 12K Flex Real-Time PCR System with the OpenArray® Platform. ***(A)*** *The volcano plot shows the relationship between the p-value and the log fold change between Treg EVs versus FoxP3^{low} T cell EVs miRNA expression levels. The dark blue line indicates the inverse log_{10} of the p-value=0.05. n=3 per group.* ***(B to D)*** *Bar graphs (mean+SEM) showing the relative quantity of* ***(B)*** *miR-142-3p,* ***(C)*** *miR-150-5p, and* ***(D)*** *miR-384-5p expressed by FoxP3^{low} T cell EVs and Treg EVs as measured by qPCR assays and normalised relative to RNU6-2. Data pooled from 3 individual experiments that were performed in technical triplicates. Statistical significance was determined using a two-tailed Student's t-test where *p<0.05 and **p<0.01.*

miRNA	Relative Quantity	p-value	Rank	Benjamini-Hochberg Procedure (i/m)Q
snoRNA202	6.453	0.006	1	0.00245098
miR-142-3p	3.633	0.020	2	0.004901961
miR-340-5p	2.017	0.042	3	0.007352941
miR-15b	3.152	0.077	4	0.009803922
miR-126	2.540	0.121	5	0.012254902
miR-152	3.458	0.122	6	0.014705882
miR-664	4.281	0.151	7	0.017156863
miR-222	3.589	0.183	8	0.019607843
miR-223-5p	3.271	0.200	9	0.022058824
let-7d	1.747	0.204	10	0.024509804
miR-685	1.443	0.251	11	0.026960784
miR-150	99.405	0.253	12	0.029411765
miR-2182	2.403	0.257	13	0.031862745
miR-200c	1.964	0.282	14	0.034313725
miR-34b-3p	4.031	0.283	15	0.036764706
miR-331	2.361	0.284	16	0.039215686
miR-132	1.999	0.285	17	0.041666667
miR-221	3.369	0.289	18	0.044117647
miR-139-5p	3.443	0.308	19	0.046568627
miR-212	2.230	0.322	20	0.049019608
miR-223-3p	3.300	0.334	21	0.051470588
miR-2138	3.840	0.336	22	0.053921569
miR-2134	4.523	0.355	23	0.056372549
miR-1198	1.532	0.388	24	0.058823529
miR-2146	2.386	0.416	25	0.06127451
miR-429	2.913	0.461	26	0.06372549
miR-328	1.604	0.473	27	0.066176471
miR-29a	1.775	0.872	28	0.068627451
miR-20b	5.306	1	29	0.071078431
miR-99b	4.508	1	30	0.073529412
miR-31	4.212	1	31	0.075980392
miR-203	3.515	1	32	0.078431373
miR-365	3.310	1	33	0.080882353
miR-362-3p	3.114	1	34	0.083333333
miR-342-3p	2.442	1	35	0.085784314
miR-155	2.427	1	36	0.088235294
miR-21	2.347	1	37	0.090686275
miR-148b	1.912	1	38	0.093137255
miR-125a-5p	1.683	1	39	0.095588235
miR-1897-5p	1.522	1	40	0.098039216
miR-744	1.462	1	41	0.100490196

Table 3.2- miRNOME result summary of the top 40 miRNA detected

Table showing the top 40 miRNAs that had higher expression levels in Treg EVs compared to FoxP3^{low} T cell EVs. The last column shows the Benjamini-Hochberg values with the formula of (i/m)Q, i= the p-value's rank, m= total number of tests and Q= the false discovery rate of 0.25

3.3.9 miR-150-5p and miR-142-3p expression levels are increased in BM-DCs following co-culture with Treg EVs

Given the high expression of miR-142-3p and miR-150-5p in Treg EVs compared to FoxP3^{low} T cell EVs (**Figures 3.8-B and C**, respectively), and that EVs can be transferred into BM-DCs (**Figures 3.6-E and F**), it led to the question of whether Treg EVs can mediate transfer of miR-142-3p and miR-150 into BM-DCs. To test this, the BM-DCs were co-cultured with Treg EVs or FoxP3^{low} T cell EVs for 24 hours at a ratio of 100 donor cell derived EVs to one recipient cell (Villarroya-Beltri, Gutiérrez-Vázquez, Sánchez-Madrid, et al. 2013). After 24 hours, BM-DCs were harvested, washed thoroughly and both miR-142-3p and miR-150-5p expression levels were measured by qPCR assays. It was observed that miR-142-3p expression was increased in BM-DCs co-cultured with Treg EVs as compared to BM-DCs cultured alone ($p<0.0001$) or with FoxP3^{low} T cell EVs ($p<0.0001$) (**Figure 3.9-A**). In addition, miR-150-5p expression levels were increased in BM-DCs co-cultured with Treg EVs as compared to BM-DCs cultured alone ($p<0.0001$) or FoxP3^{low} T cell EVs ($p<0.0001$) (**Figure 3.9-B**). This data suggested that miR-142-3p and miR-150-5p were both transferred to BM-DCs via Treg EVs.

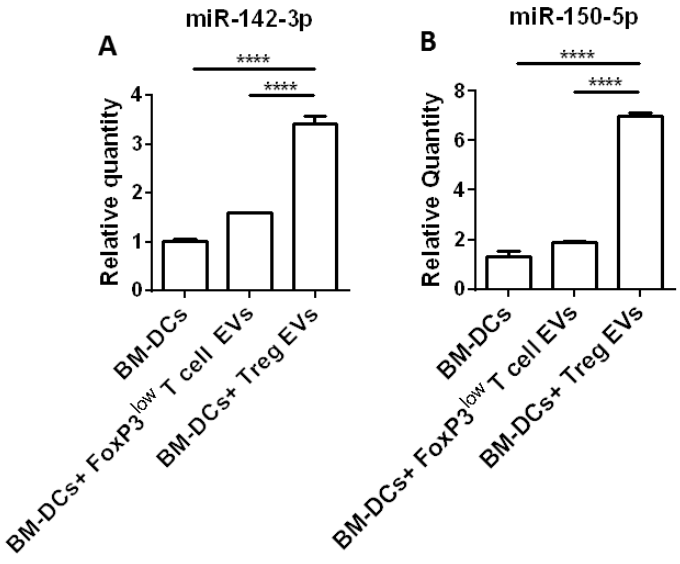


Figure 3.9- miR-150-5p and miR-142-3p expression levels are increased in BM-DCs following co-culture with Treg EVs

Bar graphs show relative quantity of ***(A)*** *miR-142-3p and* ***(B)*** *miR-150-5p expression levels in BM-DCs alone, FoxP3^{low} T cell EV-treated BM-DCs (BM-DCs+ FoxP3^{low} T cell EVs),*

*or Treg EVs-treated BM-DCs (BM-DCs+ Treg EVs). BM-DCs were treated with EVs for 24 hours, washed thoroughly and the expression levels of these miRNAs was measured by qPCR assays and normalised to RNU6-2. Data represents mean + SEM pooled from 3 individual experiments that were performed in technical triplicates. Statistical significance was determined using One-way ANOVA and Tukey's multiple comparison tests. ****p<0.0001*

However, miRNA can affect other miRNA expression levels (Matkovich 2014), and other factors could also influence miRNA expression induction or expression levels (S. Sun et al. 2017; Neilson et al. 2007; Gulyaeva and Kushlinskiy 2016; Van Wynsberghe et al. 2011). Thus to further confirm that the observed change in miRNA was due to the uptake of EVs rather than an induction of these miRNAs in the recipient cell, Dicer deficient mice, lacking mature miRNAs were used as a source of BM-DCs (Okoye et al. 2014).

Dicer gene knockout is embryonically lethal in mice (Krill et al. 2013), thus conditional knock-out $Dicer^{floxed/floxed}$ mice were used. Conditional deletion of the Dicer gene is possible as the Dicer gene was flanked by LoxP sites ($Dicer^{floxed/floxed}$) and Cre activity utilises a mutant estrogen hormone-binding domain (ER^{T}) to keep Cre inactive unless tamoxifen is present. Rosa26 locus is ubiquitously expressed in mouse cells. Thus, this mouse strain expresses Cre-ER^{T} from the ubiquitously expressed Rosa26 locus. Therefore, this mouse has an inducible deletion of Dicer in any cell that is treated with tamoxifen (Figure 2.2).

To confirm that tamoxifen reduces Dicer expression levels in BM-DCs derived from Dicer conditional knock-out ($Dicer^{-/-}$) mice, DC progenitors from bone marrow were cultured in the presence of GM-CSF and 4OH Tamoxifen or 98% ethanol (carrier/vehicle). First, the phenotypes of BM-DCs derived from these mice were assessed. As presented in Figures 3.10-A and C, 89.0±4.4% of the BM-DCs treated with 4OH Tamoxifen expressed CD11c. Of these 45.7±7.4% expressed IA^{b} (MHC class II), 82.8±7.2% were $CD80^{+}$, 56.7±10.6% were $CD86^{+}$ and 25.9±8.0% were $CD40^{+}$. The expression of all these markers were similar to BM-DCs treated with ethanol (vehicle alone). As shown in Figures 3.10-B and D, 88.1±3.0% of the BM-DCs treated with ethanol (vehicle alone)

expressed CD11c. Of these 51.8±2.6% expressed IA^b (MHCII), 87.0±4.6% were $CD80^+$, 63.7±6.5% were $CD86^+$ and 21.3±10.3% were $CD40^+$.

Next, the expression levels of miR-142-3p and miR-150-5p were assessed in BM-DCs cultured with 4OH Tamoxifen or ethanol to assess whether 4OH Tamoxifen mediated deletion of Dicer, which subsequently downregulates miRNA expression levels. The qPCR assays showed that miR-142-3p expression levels were significantly reduced by 4OH Tamoxifen treatment compared to ethanol treatment ($p=0.0448$) (Figure 3.10-E). Similar results were obtained with miR-150-5p; the expression levels of miR-150-5p were significantly reduced in 4OH Tamoxifen-treated BM-DCs compared to ethanol-treated BM-DCs ($p<0.0001$) (Figure 3-10-F). These results suggest that 4OH Tamoxifen reduced Dicer expression leading to lower miR-142-3p and miR-150-5p expression levels in the BM-DCs. Tetracycline-based reduction of gene expression is not always complete due to 'leakiness' in the system (De Veylder et al. 2000; Pham et al. 2008; Roney et al. 2016), which might explain the incomplete ablation of miRNA expression in these $Dicer^{-/-}$ BM-DCs treated with 4OH Tamoxifen. Furthermore, some miRNA generation is independent of Dicer activity (Herrera-Carrillo and Berkhout 2017; Cheloufi et al. 2010; J.-S. Yang, Maurin, and Lai 2012). In summary, BM-DCs from $Dicer^{-/-}$ mice expressed common BM-DC markers and 4OH Tamoxifen treatment did not affect BM-DC phenotype but did down-regulate the expression levels of miR-142-3p and miR-150-5p in BM-DCs.

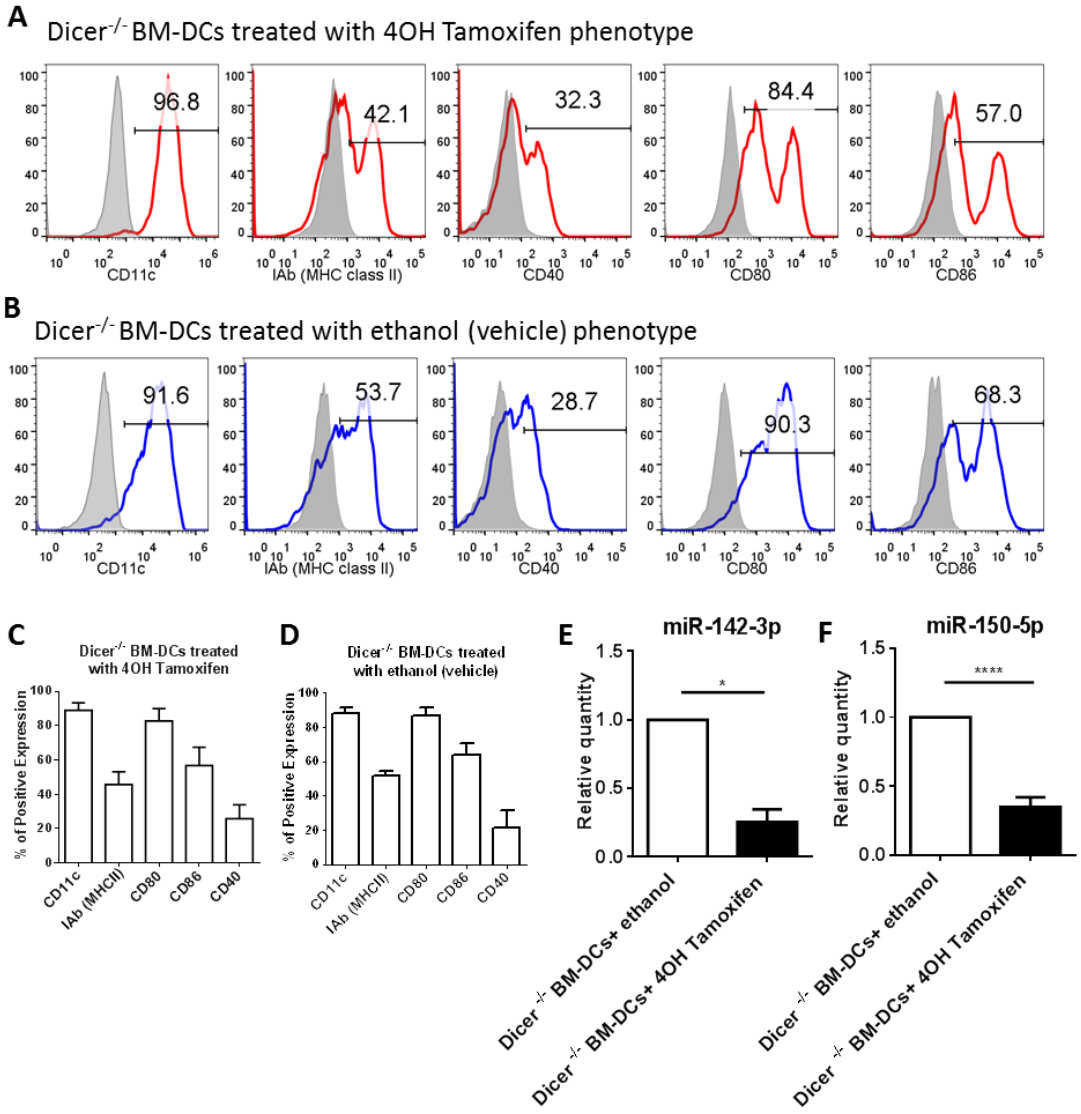


Figure 3.10- Dicer$^{-/-}$ BM-DC phenotype and reduction of miRNA expression levels

Flow cytometry histogram plots showing expression of CD11c, IA^{b} (MHC Class II), CD40, CD80 and CD86 in ***(A)*** *Dicer$^{-/-}$ BM-DCs treated with 4OH Tamoxifen (red line) or* ***(B)*** *Dicer$^{-/-}$ BM-DCs treated with ethanol (vehicle alone; blue line). Gate positions were defined on cells that were immunostained with respective isotype controls (grey solid line). The percentage of immunostained cells within the gate are indicated within each plot. The y-axis indicates the percentage of maximum expression.* ***(C and D)*** *Bar graph of pooled data of expression levels of BM-DCs markers for* ***(C)*** *Dicer$^{-/-}$ BM-DCs treated with 4OH Tamoxifen or* ***(D)*** *Dicer$^{-/-}$ BM-DCs treated with ethanol (vehicle alone). Data shown is mean+SD from 2-3 individual experiments.* ***(E and F)*** *The effect of 4OH Tamoxifen-mediated ablation of miRNA expression levels was tested for* ***(E)*** *miR-142-3p and* ***(F)*** *miR-*

*150-5p. Statistical significance was determined using Student t test. *p<0.05 and ****p<0.0001*

Next, to confirm that miR-142-3p and miR-150-5p were transferred into BM-DCs via Treg EVs rather than an induction of these miRNAs in the recipient cell, the Dicer$^{-/-}$ BM-DCs were co-cultured with Treg EVs or FoxP3^{low} T cell EVs. The co-culture of Treg EVs but not FoxP3^{low} T cell EVs with Dicer$^{-/-}$ BM-DCs resulted in an increase in miR-142-3p expression levels but this was not statistically significant (Figure 3.11-A). Nonetheless, a significant increase of miR-150-5p expression levels in Dicer$^{-/-}$ BM-DCs was observed after co-culture with Treg EVs ($p<0.0001$) but not with FoxP3^{low} T cell EVs (Figure 3.11-B). As expected, the miR-150-5p expression levels in Dicer$^{-/-}$ BM-DCs with Treg EVs treatment was significantly higher than with FoxP3^{low} T cell EVs ($p<0.0001$) (Figure 3.11-B).

In summary, these data indicated that miRNAs present in Treg EVs, namely miR-142-3p and miR-150-5p are acquired by BM-DCs.

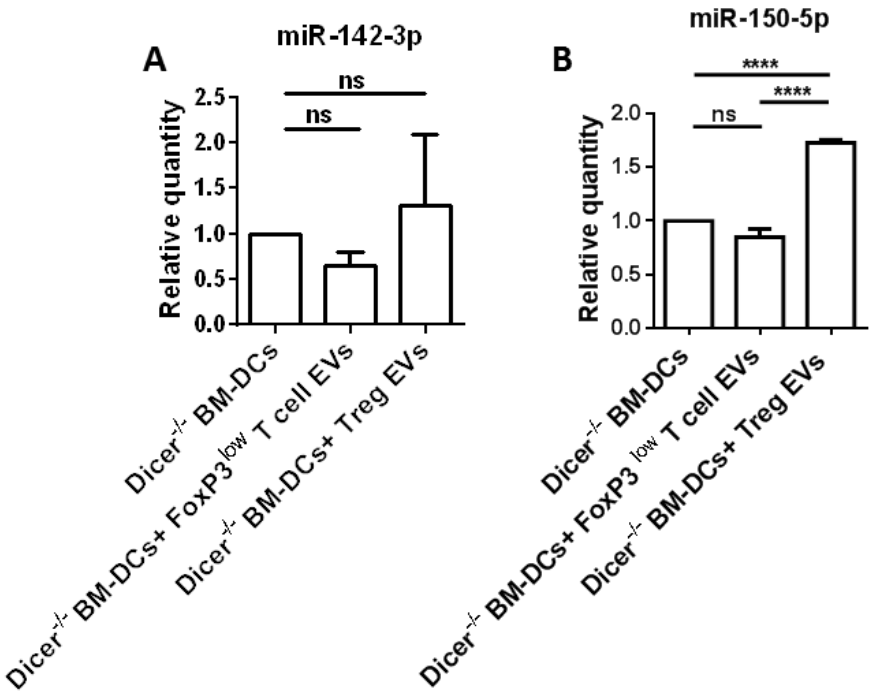


Figure 3.11- Treg EVs mediated transfer of miRNA into Dicer$^{-/-}$ BM-DCs with 4OH Tamoxifen

Bar graphs show relative quantity of **(A)** *miR-142-3p and* **(B)** *miR-150-5p expression levels in untreated Dicer$^{-/-}$ BM-DCs (Dicer$^{-/-}$ BM-DCs), FoxP3^{low} T cell EVs-treated Dicer$^{-/-}$ BM-DCs (Dicer$^{-/-}$ BM-DCs+ FoxP3^{low} T cell EVs) or Treg EVs-treated Dicer$^{-/-}$ BM-DCs (Dicer$^{-/-}$ BM-DCs+ Treg EVs). Dicer$^{-/-}$ BM-DCs were treated with EVs for 24 hours, washed thoroughly and miRNA expression levels was measured by qPCR assays and normalised to RNU6-2. Data shown is mean + SEM representative of 2 experiments that were performed in technical triplicates. Statistical significance was determined using One-way ANOVA and Tukey's multiple comparison tests. ****p<0.0001 and ns=not significant.*

3.3.10 Neither Treg EVs nor FoxP3^{low} T cell EVs affected BM-DC phenotype

Having shown that miRNAs are transferred from Treg EVs to BM-DCs, I next investigated whether this transfer affected BM-DCs phenotype. To investigate this, BM-DCs were treated with Treg EVs or FoxP3^{low} T cell EVs prior to stimulation with or without LPS and their phenotype assessed by flow cytometry. As expected LPS-stimulated BM-DCs displayed higher CD80, CD86 and CD40 expression levels compared to unstimulated BM-DCs (**Figure 3.12-A**). Neither Treg EVs nor FoxP3^{low} T cell EVs pre-treatment influenced the LPS induced expression of these (**Figures 3.12-B and C**, respectively). In summary, Tregs EVs and FoxP3^{low} T cell EVs do not affect the expression of BM-DC cell surface markers.

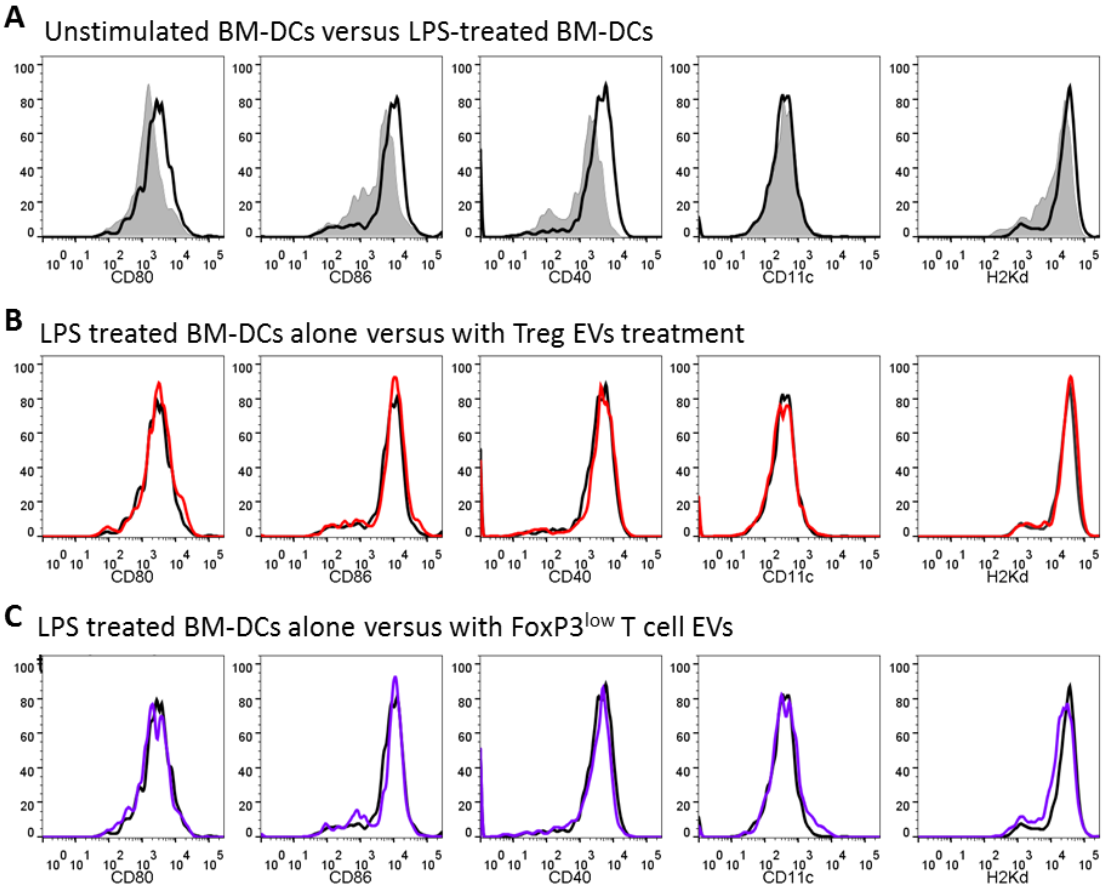


Figure 3.12- Neither Treg EVs nor FoxP3^{low} T cell EVs affect the phenotype of BM-DCs

(A) *Flow cytometry histogram plots showing the phenotype of BM-DCs that were cultured alone (grey solid line) or co-cultured in the presence of 100ng/mL LPS (black line) for the expression levels of CD80, CD86, CD40, CD11c and H2K^{d} (MHC class I). Flow cytometry histogram plots showing the phenotype of BM-DCs in the presence of 100ng/mL LPS (black line) and co-cultured with* ***(B)*** *Treg EVs (red line) or* ***(C)*** *FoxP3^{low} T cell EVs (purple line) for 24 hours. BM-DCs were washed thoroughly and subsequently cells were immunostained with fluorescently-conjugated antibodies anti-mouse specific for CD80, CD86, CD40, CD11c and H2K^{d} (MHC class I). Data is representative of one experiment. The y-axis indicates the percentage of maximum expression.*

3.3.11 Treg EVs affect BM-DCs cytokine production

Based on previous published work showing that miRNAs can modify cytokine production in DCs, including the miRNAs miR-142-3p and miR-150 (Fordham, Naqvi,

and Nares 2015; Lesley A Smyth et al. 2015) and that other EV-associated miRNAs affect target cell cytokine production (Okoye et al. 2014; Lakhter et al. 2018), I investigated whether this was the case here. Thus, in order to investigate whether EVs might affect cytokine production by BM-DCs, the cytokine production by BM-DCs with or without Treg EV or FoxP3^{low} T cell EV treatment was tested.

BM-DCs were co-cultured with or without Treg EVs or FoxP3^{low} T cell EVs and the presence and concentration of IL-6, TNF and IL-10 cytokines following exposure to LPS were tested. LPS-stimulated BM-DCs produced significantly more IL-6 ($p<0.0001$) and TNF ($p=0.0003$) compared to unstimulated BM-DCs (**Figures 3.13-A and B**, respectively). Furthermore, BM-DCs treated with LPS produced measureable levels of IL-10 compared to unstimulated BM-DCs (**Figure 3.13-C**). The additional treatment of Treg EVs or FoxP3^{low} T cell EVs to BM-DCs prior to LPS stimulation significantly reduced the production levels of IL-6 ($p<0.0001$) (**Figure 3.13-A**). Similar patterns of results were obtained when TNF production levels were measured, but the differences were not significant (**Figure 3.13-B**). In contrast, the IL-10 production levels by BM-DCs stimulated with LPS were significantly increased when BM-DCs were co-cultured with Treg EVs ($p<0.0001$) but not in the presence of FoxP3^{low} T cell EVs (no IL-10 detection). This was not due to the presence of IL-10 inside Treg EVs (data not shown) (Tung et al. 2018).

Taken together, this suggests that Treg EVs and FoxP3^{low} T cell EVs affect BM-DC cytokine production; Treg EVs reduced IL-6 whilst increasing IL-10 production levels by BM-DCs whereas FoxP3^{low} T cell EVs reduced IL-6 but did not induce IL-10 production levels. Both Treg EVs and FoxP3^{low} T cell EVs did not affect TNF production levels by BM-DCs.

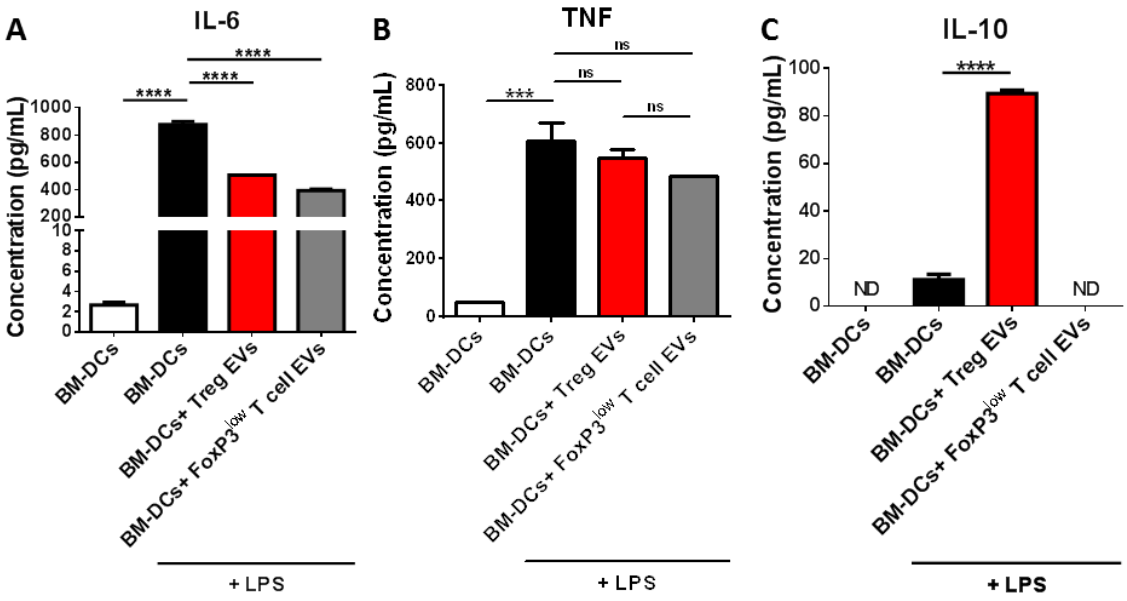


Figure 3.13- Treg EVs affect BM-DCs cytokine production

BM-DCs in the presence or absence of 100ng/mL LPS and co-cultured with or without Treg EVs or FoxP3^{low} T cell EVs for 24 hours. The supernatant from the co-cultures were harvested to test for cytokine presence and concentration using cytometric bead array and flow cytometry. The presence of ***(A)*** *IL-6,* ***(B)*** *TNF and* ***(C)*** *IL-10 cytokines from the co-cultures were assessed. Data represents 2 independent experiments that were performed in technical triplicates. Bars show mean + SEM. Statistical significance was determined using One-way ANOVA and Tukey's multiple comparison tests. ***p<0.001, ****p<0.0001, ns=not significant and ND=not detected.*

3.4 Discussion

In this thesis chapter, I confirmed previous findings that mouse Tregs release EVs (Lesley Ann Smyth et al. 2013; Okoye et al. 2014) that contained various miRNAs (Okoye et al. 2014), and that certain miRNAs were differentially expressed compared to FoxP3^{low} T cell EVs. Tregs contained miR-142-3p and miR-150-5p (Tung et al. 2018), and the same miRNAs were also present and highly expressed in their EVs compared to FoxP3^{low} T cell EVs. Treg EVs and FoxP3^{low} T cell EVs can be acquired by BM-DCs. BM-DCs increased their miR-142-3p and miR-150-5p expression levels following Treg EVs contact, suggesting that Treg EVs transferred miR-142-3p and miR-150-5p into BM-DCs. Given that miRNAs play an important role in modulating DC cytokine profiles (Lesley

A Smyth et al. 2015), I assessed whether Tregs can modulate BM-DCs via EV-associated miRNAs. BM-DCs that were in contact with Treg EVs and then stimulated with LPS have an altered cytokine production profile; IL-6 production levels were significantly reduced whereas IL-10 production levels were significantly increased. Given that miR-142-3p and miR-150-5p have been reported to affect cytokine production by target cells (Fordham, Naqvi, and Nares 2015; Lesley A Smyth et al. 2015), this suggested that it was possible that Treg EVs transferred functional miRNAs into BM-DCs that altered BM-DCs function of cytokine production to prevent a pro-inflammatory microenvironment and instead promote an anti-inflammatory milieu; hence Treg EVs may induce 'tolerogenic' BM-DCs. However, neither Treg EVs nor FoxP3^{low} T cell EVs affected the expression levels of BM-DC co-stimulatory molecules CD80 and CD86 nor BM-DC cell markers CD40 or CD11c. Noteworthy, the FoxP3^{low} T cells used in this thesis chapter originated from the Treg line and over time in culture, these cells had lost FoxP3 and CD73 expression.

3.4.1 Rab27αβ DKO Tregs

Rab27 proteins are involved in exosome release from the cell and thus Rab27αβ DKO Tregs do not release exosomes (Okoye et al. 2014). In my hands, Rab27αβ DKO Tregs were immunosuppressive in that they suppressed the proliferation of Tresps, which is in contrast to the report published by Okoye *et al.* (Okoye et al. 2014). Okoye *et al.* demonstrated that Rab27αβ DKO Tregs were not able to suppress T cell proliferation nor able to inhibit IL-2 or IFNγ cytokine production. They suggested the high dependency of exosomes in driving Treg cell-mediated suppression (Okoye et al. 2014). However, several differences in the suppression assay set up were evident in this thesis chapter compared to Okoye *et al.* (Okoye et al. 2014). Firstly these authors used freshly isolated Rab27αβ DKO Tregs, whereas in this thesis chapter Rab27αβ DKO Tregs that were expanded *in vitro* were used; secondly, the authors used plate-bound αCD3 and soluble αCD28 antibodies to stimulate T cells, whereas in this thesis chapter, APCs were used to stimulate T cells; additionally, although a minor difference was the proliferation dye used, Okoye *et al.* used CellTrace™ Violet (Okoye et al. 2014), whereas in this assay, CFDA-CFSE was used. Collectively, these differences could explain the opposing results. In particular, freshly isolated Rab27αβ DKO Tregs could act differently to *in vitro*

expanded Rab27αβ DKO Tregs, as described by Thornton and Shevach. Thornton and Shevach showed that freshly isolated Tregs were not as suppressive as *in vitro* expanded Tregs, although in that study human Tregs were used and not murine Tregs (A M Thornton and Shevach 2000). Despite Okoye *et al*. stating that Rab27αβ DKO Tregs are not suppressive, thus highlighting the great importance of exosomes for Treg suppressive functions (Okoye et al. 2014), it does query why various other suppression mechanisms that Tregs can use, such as those illustrated in **Figure 1.2,** did not play a role in suppressing T cells when the EV pathway was blocked? Given that Tregs have an extensive arsenal of suppressive mechanisms, yet not all target cell populations will be equally sensitive or affected by all Treg suppression mechanisms (Vignali 2012), suggests that Tregs maybe deploy certain selected mechanisms to suppress target cells whilst other mechanisms are not as frequently used, although this remains controversial.

3.4.2 Treg EVs and FoxP3^{low} T cell EVs are acquired by BM-DCs but their phenotype was not altered

Treg EVs and FoxP3^{low} T cell EVs are acquired by BM-DCs, which is in line with previous reports showing that human T cells mediate EV transfer of miRNA into APCs (B cells) during the immunological synapse (Mittelbrunn et al. 2011). However, it is possible that the EVs do not enter the target cell and instead only adhere to the surface of the BM-DCs. Indeed, reports have suggested that EVs can be incorporated or associated with target cells via various phagocytosis, endocytosis or receptor-ligand mediated pathways (McKelvey et al. 2015; Tian et al. 2014; Delenclos et al. 2017; Morelli et al. 2004). Many studies have found that over expression of a specific miRNA in BM-DCs did not affect cell surface markers expression while the cytokine profile was affected, so my findings are in line with published data (Lesley A Smyth et al. 2015). It is possible that cytokine modulation of BM-DCs may be a more potent form of immunosuppression compared to downregulating co-stimulatory molecules of BM-DCs. As described by Rutella *et al*., cytokine modulating tolerogenic DCs may promote *in vitro* induction of Tregs and *in vivo* prevention from post-transplantation GvHD and autoimmunity (Rutella, Danese, and Leone 2006). Thus, whether mouse

Treg EVs modified BM-DCs cytokine to induce a Treg population would be interesting to investigate.

3.4.3 Different EV isolation methods affect RNA recovery

The EV isolation method influenced the amount of RNA recovery; ExoQuick-TC™ was more efficient than ultracentrifugation at recovering EV RNA as previously reported (Y.-T. Tang et al. 2017). Treg cellular RNA and Treg EV RNA differed in their RNA profile, namely, Treg EVs and T cell EVs lacked the 18s and 28s ribosomal RNA peaks, as previously reported (Crescitelli et al. 2013; Eldh et al. 2012). As demonstrated by Crescitelli *et al*., exosomal RNA mainly consists of small RNA including mRNA and miRNA (Crescitelli et al. 2013). There are various published methods describing how to isolate and purify EVs, although no consensus has been approved as to what protocols should be followed. Nonetheless, in the EVs field, the general approach is to choose a protocol depending on what the experiment question is. For example, if EV RNA is being studied, it is more appropriate to use commercially available exosome precipitating reagents such as ExoQuick-TC™ over traditional methods such as the gold standard ultracentrifugation. The reason for choosing ExoQuick-TC™ over ultracentrifugation was due to the amount of EV RNA recovered (Y.-T. Tang et al. 2017), which could also reflect on the EVs recovery obtained from using this method. Therefore, I compared the ultracentrifugation and ExoQuick-TC™ methods of EV RNA isolation. Possible explanations of the observed results could be due to the notion that (i) ultracentrifugation does not recover the majority of EVs during processing (ii) ExoQuick-TC™ precipitates 'free' (not bound to EVs) miRNA (iii) ExoQuick-TC™ precipitates proteins which miRNA may be bound to. Although careful steps were taken to remove contaminating cells and cell debris before the EVs isolation step, it cannot be completely excluded that the possibility of contaminating proteins could have been isolated together with EVs.

3.4.4 miRNA modulate cytokine production

Multiple miRNAs have been linked to the inhibition of pro-inflammatory cytokine production by DCs (Lesley A Smyth et al. 2015). Examples of this include miR-21, which inhibits IL-12p35 production (T. X. Lu, Munitz, and Rothenberg 2009) and miR-

148/miR-152 which suppresses the production of both IL-6 and IL-12 cytokines (X. Liu et al. 2010). miR-21 was expressed in higher levels in Tregs compared to BM-DCs (Tung et al. 2018). Furthermore, miR-21, miR-148 and miR-152 were found present in the Treg EVs from the miRNOME screen. But I chose to focus on miR-142-3p and miR-150-5p because these two miRNAs had a significantly higher expression level in Treg EVs compared to FoxP3^{low} T cell EVs. Both of these also have cytokine modulating properties as explained below.

Ectopic expression of miR-142-3p in human DCs leads to decreased production of IL-12, IL-6 and TNFα after TLR ligation (Fordham, Naqvi, and Nares 2015; Naqvi, Fordham, and Nares 2015). In mouse DCs, miR-142-3p directly targets the 3'UTR of IL-6 mRNA (Y. Sun et al. 2011). In 2013, Sun *et al.* transfected splenic CD11c^{+} murine DCs with pre-miR-142-3p and observed reduced endogenous levels of IL-6. On the other hand, knocking down miR-142-3p by transfecting DCs with a miR-142-3p "locked nucleic acid" (LNA) probe resulted in increased IL-6 production following LPS activation. This study also demonstrated miR-142-3p did not affect IL-10 cytokine levels post LPS activation (Y. Sun et al. 2011).

King *et al*. demonstrated that miR-150 expression in human CD4^{+} T cells regulates IL-10 production. Knockdown of miR-150 expression levels in primary T cells resulted in reduced IL-10 production levels following activation with anti-CD3 and CD46 antibodies (B. C. King et al. 2016). Given these findings I suggest that transferring both miR-150-5p and miR-142-3p may be linked to the increased IL-10 and decreased IL-6 production levels, respectively, observed in BM-DCs treated with Treg EVs.

Whether acquisition of other miRNAs, such as miR-21, also present in the Treg EVs resulted in production of IL-10 following TLR ligation requires further studies. miR-21 has been shown to indirectly affect IL-10 production. Transfection of pro-miR-21 into RAW264.7 cells led to increased IL-10 production following LPS activation. The authors of this data concluded that LPS induced miR-21 which targets PDCA4, a negative regulator of IL-10, leading to increased IL-10 production (Sheedy et al. 2010).

Notably, miR-384-5p was significantly lower expressed in Treg EVs compared to FoxP3^{low} T cell EVs. Qu *et al.* reports that miR-384 regulated the Th17/Treg balance during EAE pathogenesis (Qu et al. 2017). These authors demonstrated that overexpression of miR-384 *in vivo* resulted in exacerbated EAE conditions. miR-384 promoted the differentiation of naive T cells into Th17 cells which produced elevated levels of pro-inflammatory IL-17 cytokine. The observation that Treg EVs expressed significantly lower levels of miR-384-5p compared to FoxP3^{low} T cell EVs may be a possible mechanism to inhibit the induction of Th17 cells and instead promote Treg differentiation.

It is possible that the modification of BM-DCs by Treg EVs may complement or aid the other pathways as outlined in section 1.6.3, used by Tregs to induce a 'tolerant' APC. Given that previous studies have used rat Treg EVs to induce tolerance in the setting of transplantation (X. Yu et al. 2013; Aiello et al. 2017) it is possible that the EVs is one of the major pathways by which Tregs function through modification of cytokines released by BM-DCs by transferring miRNAs.

3.4.5 Other miRNA functions

miR-142-3p can also attenuate phagocytosis. Overexpression of a miR-142-3p mimic in primary human monocytes and DCs reduced *E.coli* fluorescent OVA as well as antibody-mediated phagocytosis, compared to controls (Naqvi, Fordham, and Nares 2015; Naqvi et al. 2016). Overexpression of miR-142-3p in human DCs has been shown to affect at least 40 genes, some of which are associated with phagocytosis as well as cell signalling associated genes such as PKCα and cell mobility such as N-Wasp (Naqvi, Fordham, and Nares 2015). Overexpression of miR-142-3p in DCs has also been linked with decreased T cell activation, which was independent of any changes to MHC or co-receptor expression on the DCs (Naqvi et al. 2016). However, overexpression of this miRNA did not affect MHC class II, CD80 or CD86 expression levels compared to control and non-manipulated DCs (Naqvi et al. 2016), which I have also demonstrated with the treatment of Treg EVs (which contained miR-142-3p) also did not alter the expression levels of CD80 nor CD86 by BM-DCs. Taken these together the observation that miR-142-3p is increased in BM-DCs following exposure

to both Tregs and their EVs, I suggest that miRNA transfer by Tregs may play a key functional role in their suppressive function with respect to APCs function.

Several studies have shown that miRNAs I found present in FoxP3^{low} T cell EVs and Treg EVs directly affect validated target molecules present within the recipient cell. For example, c-Myb mRNA, an identified target for miR-150-5p, was downregulated in a miR-150-5p negative hepatocyte derived cellular carcinoma cell line following exposure to human Treg EVs containing this miRNA (Torri et al. 2017). It is therefore feasible that the miRNAs present in these EVs will affect their mRNA target.

EVs have been shown to contain many other types of RNA species in addition to miRNAs some of which may modify cytokine expression. miRNAs constitutes only a small fraction of small RNAs found in immune and non-immune cell derived EVs and other RNA species have been identified including, snoRNA, piRNA, lincRNA, rRNA, tRNA (Nolte-'t Hoen et al. 2012) and Y RNA (Nolte-'t Hoen et al. 2012; Cambier et al. 2017). Indeed, snoRNA202 was observed in the Treg EVs. Recently, Y RNA, identified in cardiosphere-derived cell exosomes, was linked to IL-10 expression and secretion (Cambier et al. 2017). Transfection of macrophages with this Y RNA led to increased production of this cytokine. Whether other RNA species are present in Treg EVs is out with the scope of this PhD project, however given the alterations to cytokine production observed in BM-DCs following treatment with Treg EVs it is possible that the changes observed are mediated by the transfer of a combination of RNA species or indeed a combination of miRNAs and cell surface proteins.

In 2013, Bryniarski *et al*. published the first study demonstrating that CD8$^+$ suppressor T cells release exosomes-like EVs that encompassed miR-150 and delivered it to Teffs in an antigen-specific manner. The exosomes-like EVs were primed to be antigen specific via a surface coating of antibody light chains, hence allowing the EVs a selective uptake by Teffs. The targeted delivery of the inhibitory miR-150 into Teffs resulted in inhibition of their cellular activity (Bryniarski et al. 2013). Bryniarski *et al.* went on to demonstrate that these EVs were able to function at the *in vivo* setting to inhibit contact sensitivity (Bryniarski et al. 2013).

3.4.6 Let-7d

Similar to Okoye *et al*. (Okoye et al. 2014), we also found the presence of Let-7d in the Treg EVs, even though this was higher expressed in Treg EVs compared to FoxP3^{low} T cell EVs this was not statistically significant from the miRNOME screen. However, given that Let-7d was shown to be the relevant miRNA involved in driving the immunosuppressive ability of Treg EVs in *in vitro* and *in vivo* settings, it raises the possibility that Let-7d in these Treg EVs used in this thesis chapter may also similarly be involved in immunosuppression.

Transfer of several miRNAs with the potential to induce non-inflammatory cytokine release by BM-DCs may help create a 'tolerogenic' environment, although whether the same occurs *in vivo* and how this is modulated in autoimmune conditions has yet to be elucidated. This thesis chapter results could provide valuable information regarding the modification of Tregs for therapeutic use. Given that Tregs are currently used in clinical trials (Bluestone et al. 2015), creating Tregs that deliver miRNAs to DCs via EVs may help increase the efficacy of this therapy in patients receiving a transplant or with autoimmune diseases.

3.5 Summary

In summary, in this chapter I have shown that mouse Tregs release EVs that contain higher expression levels of miR-142-3p and miR-150-5p compared to FoxP3^{low} T cell EVs. BM-DCs acquired miR-142-3p and miR-150-5p from Treg EVs leading to an altered BM-DCs cytokine production profile; from a pro-inflammatory microenvironment to an anti-inflammatory milieu; IL-6 was reduced whereas IL-10 was increased. These cytokine alterations may be related to miR-142-3p and miR-150-5p contained in Treg EVs. Hence Treg EVs may induce 'tolerogenic' BM-DCs. However, neither Treg EVs nor FoxP3^{low} T cell EVs altered the expression of DC co-stimulatory molecules CD80, CD80 nor DC cell markers CD40 or CD11c.

Chapter 4
Characterisation of Human Treg EVs *in vitro*

Chapter 4 - Characterisation of Human Treg EVs *in vitro*

4.1 Introduction

4.1.1 Human T cell derived EVs and their characterisation so far

The full characterisation of human Tregs heterogeneity, phenotype and function is currently incomplete. Understanding the precise mechanisms of action of Tregs is important not only to shed some light into their biological role but also for optimising their use in the clinical setting. I have shown in Chapter 3 that murine Tregs produce EVs that regulate BM-DCs function, we know also from previous work that Treg EVs can target other cells, such as T cells directly (Lesley Ann Smyth et al. 2013; Okoye et al. 2014; Aiello et al. 2017; X. Yu et al. 2013; Torri et al. 2017; Azimi et al. 2018).

T cells releasing EVs has been well documented since many years ago (Ventimiglia and Alonso 2016). For example, a study in 2002 by Blanchard *et al.* demonstrated that human T cell derived exosomes contain the TCR/CD3/ζ complex which provided T cell exosomes with the potential to deliver activation signals to cells bearing the complementary peptide/MHC complexes (Blanchard et al. 2002).

In 2013, my host laboratory had published the first evidence that mouse Tregs release EVs (Lesley Ann Smyth et al. 2013), and in a review published in 2014, my host laboratory had stated that we had evidence that human Tregs also produced these vesicles (Agarwal et al. 2014). I had obtained the data to demonstrate the novel finding that human Tregs release EVs which were immunomodulatory (presented in this thesis chapter), however, since the commencement of my PhD studies, this novel finding had recently been published by two other groups in 2017 (Torri et al. 2017) and in 2018 (Azimi et al. 2018).

Torri *et al.* isolated ~150nm sized and cup-shaped EVs derived from Th1, Th17 and Tregs; the authors reported that Treg EVs, but not Th1 EVs or Th17 EVs, suppressed $CD4^+$ T cell proliferation (Torri et al. 2017), which the authors attributed to specific miRNA content (see section 1.7.8.3 for more details). Azimi *et al.* (Azimi et al. 2018) confirmed the findings of Torri *et al*. (Torri et al. 2017) that human Treg exosomes are immunomodulatory. Azimi *et al.* compared human Treg exosomes derived from healthy controls and MS patients and found that both types of exosomes were suppressive and

induced apoptosis of conventional T cells, but the MS Treg exosomes were less potent than that of healthy controls (Azimi et al. 2018), suggesting that MS Treg exosomes were impaired.

4.1.2 Time course release of EVs

The presence of CD63 and CD81 are markers commonly used to define EVs (Clotilde Théry, Zitvogel, and Amigorena 2002; Colombo, Raposo, and Théry 2014). The expression levels of CD63 and CD81 on the cell surfaces may be very low but their intracellular levels are comparatively higher as CD63 and CD81 are highly enriched in the endosomal compartments (Kobayashi et al. 2000; Das and Pellett 2011; Piper and Luzio 2001). To date, the intracellular expression levels of CD63 or CD81 in Tregs (compared to other T cells) is unclear and thus remains to be further clarified.

It has been previously reported that T cells (Blanchard et al. 2002) and Tregs (Okoye et al. 2014; Lesley Ann Smyth et al. 2013) at steady state release very low numbers of EVs, but their activation increases the numbers of EVs released. Most research groups activate T cells and Tregs via their TCR and subsequently collect the EVs from their culture supernatant (Tung et al. 2018; Lesley Ann Smyth et al. 2013; Okoye et al. 2014; Torri et al. 2017). However, at the time that I started the analysis of Treg EVs, a time-course of EVs collection ranging from 24 to 48 hours post cell activation had not been done with human Tregs. Given that human Tregs release EVs which may be dependent on Ca^{2+} influx, like in mouse Tregs (Okoye et al. 2014), it has not been addressed whether these human Tregs continually release EVs until their Ca^{2+} levels decline back to normal levels. Furthermore, a direct comparison of the number of EVs released by Tregs and autologous Teffs has also not been reported in literature and remains to be determined. Given the human to human variation in cell responses, it would be important to monitor the release of EVs from both cell types derived from the same individual. Furthermore, although in the mouse Treg setting, Okoye *et al*. found that Tregs released more $CD63^+$ EVs than $CD4^+$ T cells (Okoye et al. 2014), whether this is also the case for human Tregs and Teffs has not been shown and remains to be elucidated.

Another important point is to understand how human Treg EVs affect Tresps responses and whether they regulate for example cytokine production by Tresps. Given that murine Treg EVs significantly decreased the production levels of pro-inflammatory cytokines IFNγ and IL-2 (Lesley Ann Smyth et al. 2013; Okoye et al. 2014), it would be possible that human Treg EVs may also function similarly and thus warranted further investigation.

4.2 Aims and Objectives

Aim

The aim of this chapter was to investigate whether human Tregs expanded following a protocol used for Treg therapy produced EVs and if the answer was positive to assess whether they had the ability to inhibit Tresps proliferation and modulate cytokine production.

Objectives:

(1) Phenotype Human Tregs and Teffs using common T cell markers and also markers commonly used to define EVs such as CD63, CD81 and CD9
(2) Enumerate EV frequency before cell activation and 24 hours and 48 hours post-cell activation
(3) Characterise human Treg EVs by EM, NanoSight analysis, and evaluate CD63 and CD81 expression on EVs
(4) Study the immunomodulatory function of Treg EVs and Teff EVs and investigate whether they alter the ability of Tresps to produce cytokines

4.3 Results

4.3.1 Phenotypic characterisation of human Tregs and Teffs

$CD4^+CD25^+$ human Tregs or autologous $CD4^+CD25^-$ Teffs were isolated from the buffy coat of healthy donor peripheral blood using an established GMP-compatible protocol (Scottà et al. 2013). Using this approach, the percentage of cells expressing $CD4^+CD25^+$ (Tregs) was 95.1% ± 3.02% (**Figures 4.1-A and B**) and $CD4^+CD25^-$ (Teffs) was 93.9% ± 2.01% (**Figures 4.1-C and D**). Isolated human Tregs and Teffs were expanded *ex vivo,* polyclonally in the presence of activation beads and IL-2. Tregs received the addition of rapamycin, which selectively promotes the expansion of $CD4^+CD25^+FoxP3^+$ Tregs whilst minimising the expansion of any contaminating Teffs population (Fort and Narayanan 2010; Golovina et al. 2011; Scottà et al. 2013; Battaglia et al. 2006). Following expansion, the phenotype of Tregs and Teffs was assessed. Expanded human Tregs exhibited a typical phenotype; the percentage of cells expressing the following markers was; $CD3^+$ (98.7% ± 0.5%), $TCR\alpha\beta^+$ (99.8% ± 0.1%), $CD4^+$ (97.1% ± 0.6%), $CD25^+$ (91.7% ± 1.2%), $CD127^{low}$ (21.4% ± 5.5%), $FoxP3^+$ (88.2% ± 2.8%), $CTLA4^+$ (79.3 ± 3.6%), $CD39^+$ (86.3% ± 3.9%) and $CD73^+$ (47.5% ± 9.4%) (**Figures 4.2-A and B**).

In addition, human Tregs expressed common markers used to define EVs, these included CD63, CD81 and CD9 (Colombo, Raposo, and Théry 2014; Clotilde Théry, Zitvogel, and Amigorena 2002) which its expression were found both on the cell surface and intracellularly. The latter was anticipated given that CD63, CD81 and CD9 are found on endosomes and endocytic compartments (Piper and Luzio 2001; Das and Pellett 2011). The percentage of Tregs expressing cell surface CD81 was high (98.7% ± 0.4%) and higher than the percentage of cells expressing CD63 or CD9 (27.6% ± 8.4% and 28.02% ± 5.4%, respectively) (**Figures 4.2-C and D**). The percentage of cells expressing total (cell surface and intracellular) CD63 and CD81 were high (92.8% ± 4.3% and 98.8% ± 0.2%, respectively) although for CD9, this was comparatively lower (30.8% ± 10.9%) (**Figures 4.2-C and D**).

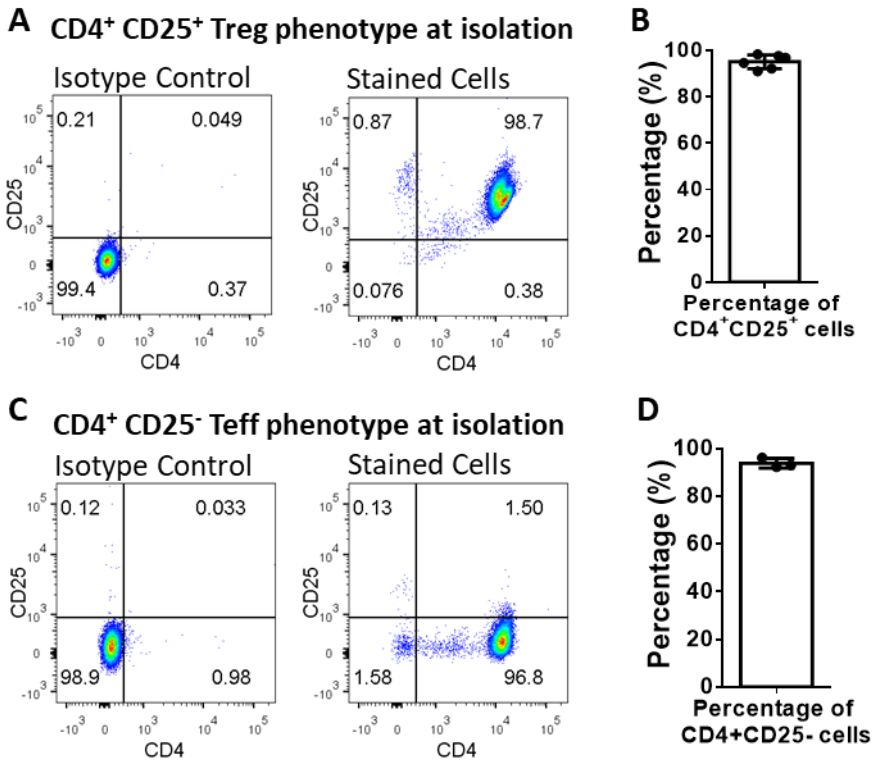


Figure 4.1- Human Tregs and Teffs phenotype at isolation

Tregs and Teffs were freshly isolated from the buffy coat of healthy donor peripheral blood and subsequently stained for anti-human CD4 and CD25 antibodies or relevant isotype controls. Flow cytometry dot plots show the phenotype of stained ***(A)*** *Tregs and* ***(C)*** *Teffs. Bar graph for* ***(B)*** *Tregs n= 6 and* ***(D)*** *Teffs n=3. Representative flow cytometry dot plots are shown.*

Teffs were also phenotypically assessed following expansion. As expected, human Teffs exhibited a phenotype that was similar to Tregs, but the percentages of cells expressing defined markers were different. The percentages of human Teffs which expressed the following markers were; $CD3^+$ (94.6% ± 4.1%), $TCR\alpha\beta^+$ (99.8% ± 0.1%), $CD4^+$ (93.8% ± 1.5%) and $CD127^{low}$ (28.3% ± 7.1%) (**Figures 4.3-A and B**). Although the Teffs were also $CD39^+$ (55.6% ± 10.2%) (**Figures 4.3-A and B**), a lower percentage of cells expressed this molecule compared to Tregs. In comparison to the Tregs, the percentage of Teffs were noticeably $CD25^{low}$ (23.7% ± 5.4%), $FoxP3^{low}$ (6.1% ± 1.0%), $CTLA4^{low}$ (15.8% ± 3.4%) and $CD73^{low}$ (18.7% ± 6.4%) (**Figures 4.3-A and B**).

A high percentage of Teffs expressed cell surface CD81 (96.5 ± 1.4%), however, a comparably lower number of cells expressed CD63 and CD9 (6.9% ± 2.6% and 32.3% ± 14.0%, respectively) (Figures 4.3-C and D). A high percentage of Teffs expressed total CD63 and CD81 (87.0% ± 9.8% and 90.0% ± 2.8%, respectively) although the percentage of cells expressing CD9 was comparatively lower (15.5% ± 3.2%) (Figures 4.3-C and D).

Overall, human Tregs and Teffs expressed markers commonly used to define them. The cell surface and intracellular expression of endosomal markers CD63 and CD81 was present in the large majority of Treg and Teffs.

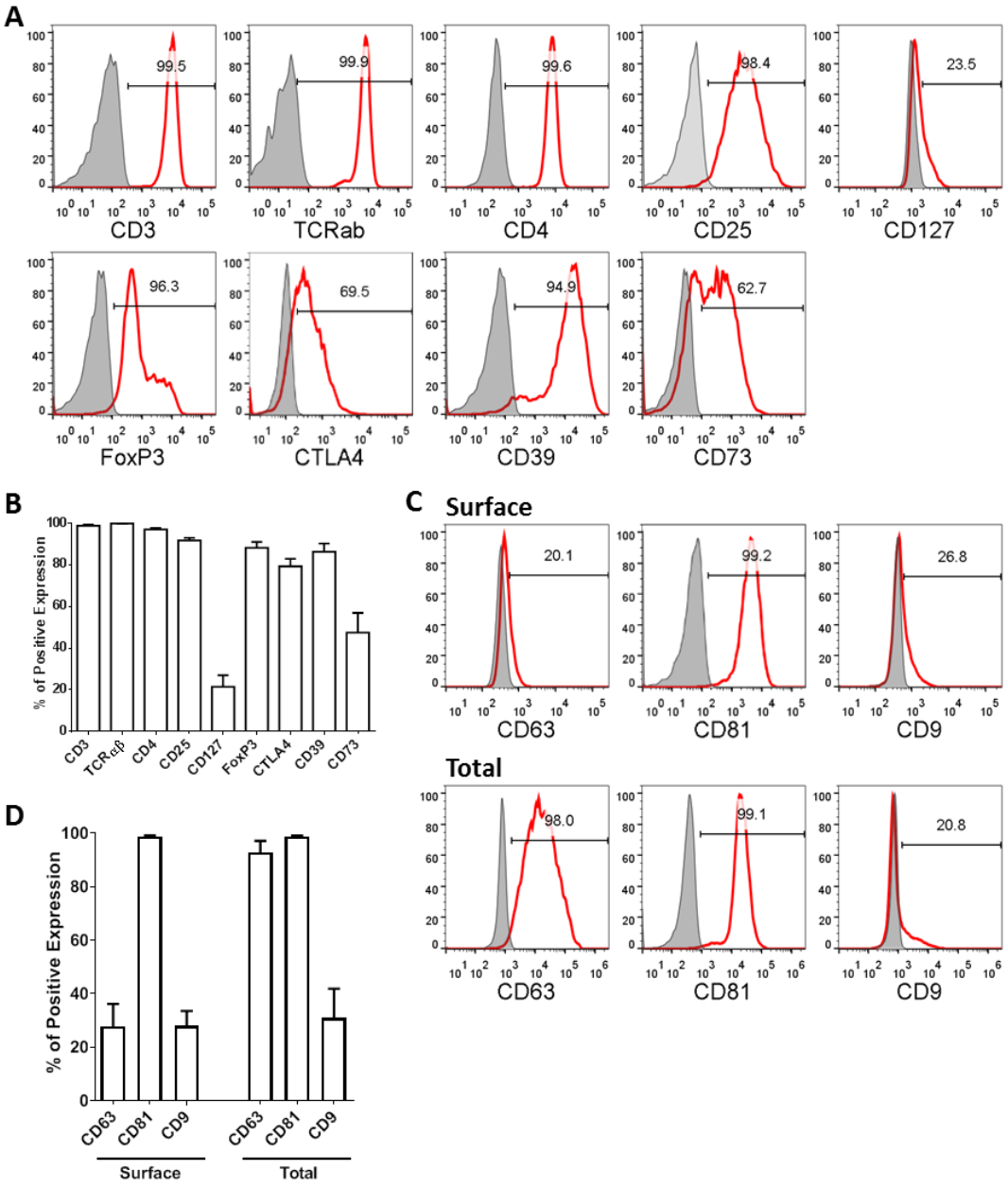


Figure 4.2- Phenotype of Human Tregs

Tregs were expanded with anti-CD3/CD28 beads, rapamycin and IL-2. ***(A)*** *Tregs were assessed for the expression of the following markers by flow cytometry by staining for anti-human anti-CD3; TCRαβ; CD4; CD25; CD127; FoxP3; CTLA-4; CD39 and CD73. Data shown are representative FACS plots from 3-12 independent experiments. The y-axis indicates the percentage of maximum expression.* ***(B)*** *Pooled flow cytometry data showing the percentage of positive expression levels of individual markers. Bars show the mean + SEM pooled from 3-12 individual experiments.* ***(C)*** *The surface and total expression of CD63, CD81 and CD9 were assessed in Tregs. The y-axis indicates the percentage of maximum expression.* ***(D)*** *Pooled flow cytometry data showing the*

percentage pf positive expression of surface or total CD63, CD81 and CD9. Bars show the mean + SEM pooled from 3-14 individual experiments.

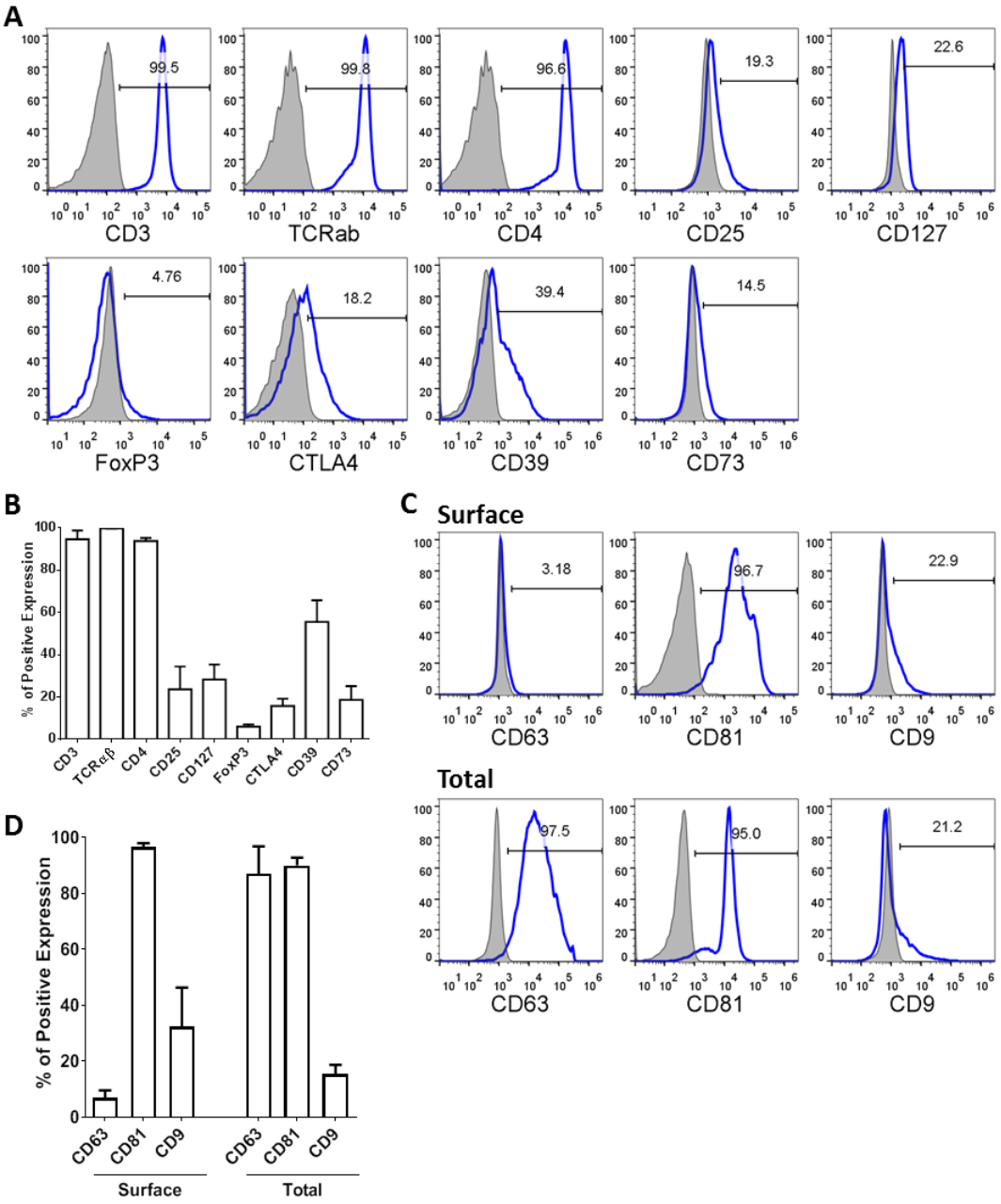


Figure 4.3- Phenotype of Human Teffs

Teffs were expanded with anti-CD3/CD28 beads and IL-2. ***(A)*** *Teffs were assessed for the expression of the following markers by flow cytometry by staining with anti-human anti-CD3; TCRαβ; CD4; CD25; CD127; FoxP3; CTLA-4; CD39 and CD73. Data shown are representative FACS plots from 3-5 independent experiments. The y-axis indicates the percentage of maximum expression.* ***(B)*** *Pooled flow cytometry data showing the percentage of positive expression of T cell markers. Bars show the mean + SEM pooled*

from 3-5 individual experiments. ***(C)*** *The surface and total expression of CD63, CD81 and CD9 were assessed in Teffs. The y-axis indicates the percentage of maximum expression.* ***(D)*** *Pooled flow cytometry data showing the expression levels of surface or total CD63, CD81 and CD9. Bars show the mean + SEM pooled from 3-5 individual experiments.*

4.3.2 Activated Human Tregs and Teffs release extracellular vesicles

As mentioned in section 1.7.8, murine Tregs (Lesley Ann Smyth et al. 2013; Tung et al. 2018; Okoye et al. 2014; Aiello et al. 2017; X. Yu et al. 2013) and human T cells (Blanchard et al. 2002) release EVs upon TCR engagement. During my PhD studies another group published in 2017, the first evidence that freshly isolated human Tregs produce EVs (Torri et al. 2017), strengthening our preliminary findings, which are described below.

To validate the release of EVs, Tregs or Teffs were stimulated with plate bound anti-CD3/CD28 antibodies for 24 hours, and activation was assessed and confirmed by measuring the upregulation of the T cell activation marker CD69 (Figures 4.4-A and B). In order to confirm the presence of EVs released from the activated Tregs, EVs isolated from the supernatant of Tregs were examined under EM. Figure 4.4-C shows a representative heterogeneous population of Treg EVs with a cup-shape morphology that was observed, and previously described by others (Colombo, Raposo, and Théry 2014). The presence of EVs was further analysed by NanoSight analysis which demonstrated that Treg EVs had a mode of 126nm ± 5.9nm and a mean size of 160nm ± 7.1nm (Figure 4.4-D). Similar analysis with Teff EVs revealed that they had a mode of 128nm ± 6.9nm and a mean size of 180nm ± 12.8nm (Figure 4.4-E). A representative image of the NTA multi-parameter output of intensity, diameter and concentration of particle measurement is shown in Figure 4.4-F. Due to restricted access to the Centre for Ultrastructural Imaging, King's College London, it was not possible to analyse the Teff EVs using EM.

In order to confirm that TCR activation increased the numbers of released EVs, EVs derived from unstimulated cells and activated cells were compared on NanoSight. The number of released EVs increased significantly after cell activation compared to unstimulated cells. For Tregs, an average of 1151 EVs (range of 554 – 1828 EVs) was

released per activated Treg cell (Figure 4.4-G). However, Teffs released considerably more EVs than Tregs, which on average was 2514 EVs (range of 1884 – 3568 EVs) released per activated Teff cell (Figure 4.4-H and I).

To investigate whether cells activated for an extended period of time released higher numbers of EVs, the EV numbers were enumerated by NanoSight after 0, 24 or 48 hours post-cell activation. Tregs released significantly higher numbers of EVs after 48 hours compared to 0 or 24 hours post-cell activation (p= 0.0336 and p= 0.0310, respectively) (Figure 4.4-J). In a similar manner, Teffs also released higher numbers of EVs after 48 hours compared to 0 or 24 hours of post-cell activation (Figure 4.4-K), however, only 2 samples were used for the NanoSight readings and thus no statistical test was performed.

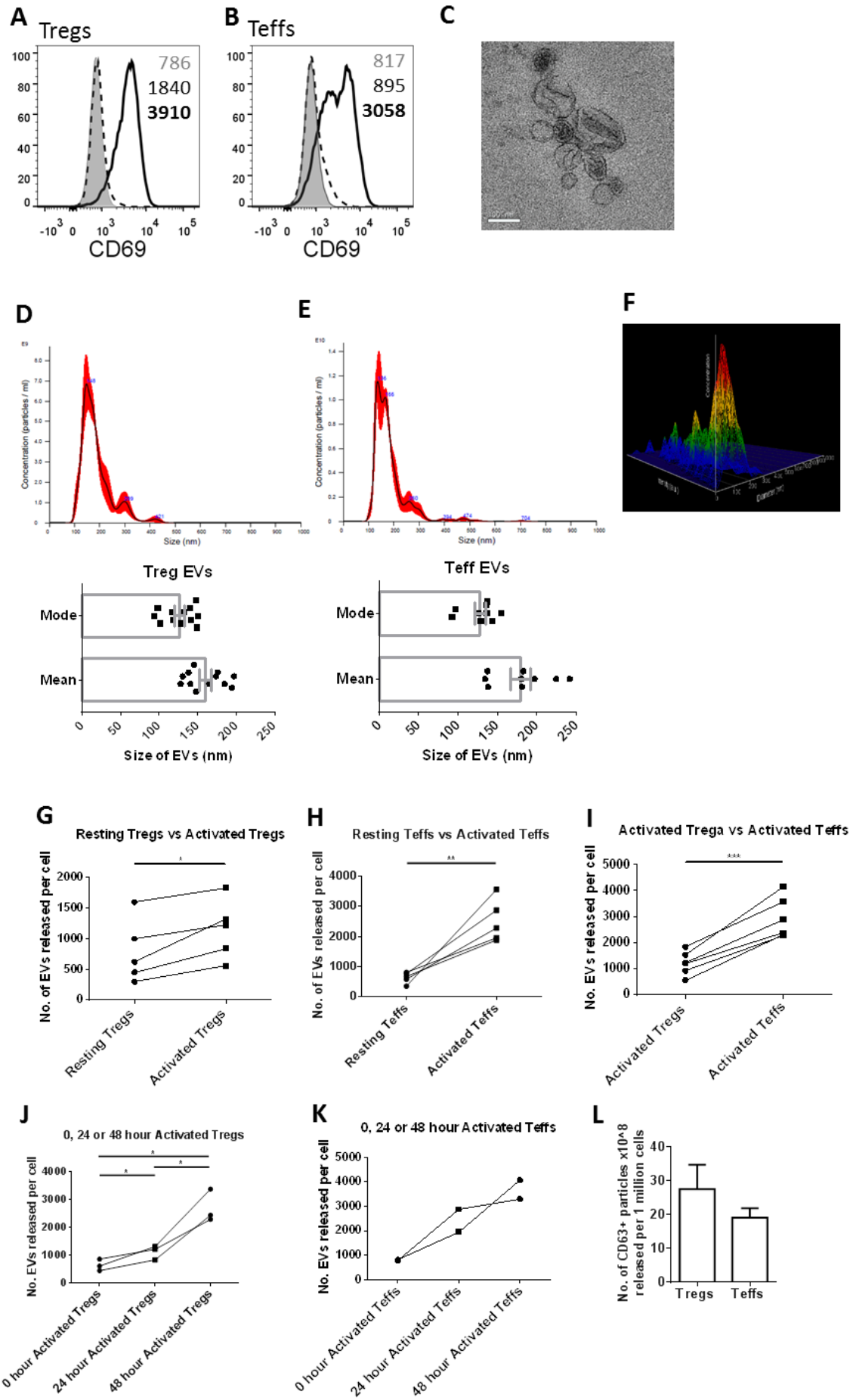
It has been suggested that cell culture conditions can influence the types of EVs produced (Gudbergsson et al. 2016). It is well documented that Tregs require IL-2 for survival (Scottà et al. 2013), however IL-2 was not added to the culture medium when Tregs were activated for 24 hours and 48 hours. The reason for not adding IL-2 to the culture medium was to avoid the presence of IL-2 in the EV preparations as this could have induced the proliferation of Tresps and masked any effect of the EVs in downstream applications. However, to avoid Treg death, due to the absence of IL-2, and the subsequent release of apoptotic bodies which can be of a similar size range and express CD63 (Crescitelli et al. 2013), therefore making it difficult to separate non-apoptotic EVs from apoptotic bodies, it was decided that 24 hours derived EVs should be used instead of 48 hours derived EVs for all the subsequent assays.

Given that Okoye *et al*. showed in the mouse setting, Tregs released more $CD63^+$ exosomes than $CD4^+$ T cells (Okoye et al. 2014), the EVs released by human Treg and Teffs were evaluated by NanoSight. However, NanoSight does not discriminate between $CD63^+$ and non-$CD63^+$ EVs but rather measures total EV numbers, thus a CD63 ExoELISA™ Ultra kit was used to enumerate $CD63^+$ particles. Indeed, human Tregs had a trend to release more $CD63^+$ EVs compared to Teffs (Figure 4.4-L). However, because only 2 donor samples were used for the CD63 ExoELISA™ Ultra kit, a statistical test was not performed. Therefore, despite Teffs releasing more total EV numbers than Tregs, Tregs released more $CD63^+$ EVs than Teffs.

To further address how many EVs were CD63 positive and how many were negative for CD63, ImageStream® analysis of Treg EVs was employed (Headland et al. 2015). Treg EVs were double stained with CFDA-CFSE and CD63. It was clear from the results that not all Treg EVs were $CD63^+$ (46% of EVs were $CD63^+$) (Figure 4.4-M), given the heterogeneity of EVs (Willms et al. 2018; Ferguson and Nguyen 2016; Willms et al. 2016), it is therefore appreciable that EVs may instead express CD81 or CD9 (Clotilde Théry, Zitvogel, and Amigorena 2002; Colombo, Raposo, and Théry 2014; Graça Raposo and Stoorvogel 2013). Indeed, another report demonstrated CD81 and CD9 presence on exosomes but not CD63 (Saunderson et al. 2008), further confirming the heterogeneity of EVs.

Exosomes and small EVs are too small in size (50-150nm) to be detected directly on a conventional flow cytometer, although for larger EVs this is possible. However, by adhering small EVs to 4µm aldehyde/sulphate latex beads their surface area is increased thus enabling phenotyping via flow cytometry (Lesley Ann Smyth et al. 2013). To investigate whether EVs expressed other Treg-related markers, these particles were bound onto latex beads and stained for the EV markers CD63 and CD81 along with other markers found on Tregs. The particles displayed typical EV markers CD63 and CD81, as expected (Figure 4.4-N). Additionally, Treg-associated markers, namely CD25 and CD39 molecules were also found expressed by some of the EVs. However, EVs were negative for the expression of CD4, CD73 and CTLA4 (Figure 4.4-N). Due to time limits on the PhD project and as the main focus of this project was Treg EVs, rather than Teff EVs, the latter were not phenotyped.

Overall, activated human Tregs and Teffs released vesicles that are approximately 126nm in size, with significantly more EVs released 24 and 48 hours post-cell activation, although I cannot exclude that some were apoptotic bodies at the latest time-point tested. These Treg EVs expressed EV markers CD63 and CD81, although not all EVs expressed these. Furthermore, Treg EVs contained Treg cell-associated proteins CD25 and CD39.


A
Tregs
786
1840
3910
CD69
B
Teffs
817
895
3058
CD69
C
D
Concentration (particles / ml)
Size (nm)
E
Concentration (particles / ml)
Size (nm)
F
Treg EVs
Mode
Mean
Size of EVs (nm)
Teff EVs
Mode
Mean
Size of EVs (nm)
G
Resting Tregs vs Activated Tregs
No. of EVs released per cell
Resting Tregs
Activated Tregs
H
Resting Teffs vs Activated Teffs
No. of EVs released per cell
Resting Teffs
Activated Teffs
I
Activated Trega vs Activated Teffs
No. EVs released per cell
Activated Tregs
Activated Teffs
J
0, 24 or 48 hour Activated Tregs
No. EVs released per cell
0 hour Activated Tregs
24 hour Activated Tregs
48 hour Activated Tregs
K
0, 24 or 48 hour Activated Teffs
No. EVs released per cell
0 hour Activated Teffs
24 hour Activated Teffs
48 hour Activated Teffs
L
No. of CD63+ particles x10^8 released per 1 million cells
Tregs
Teffs

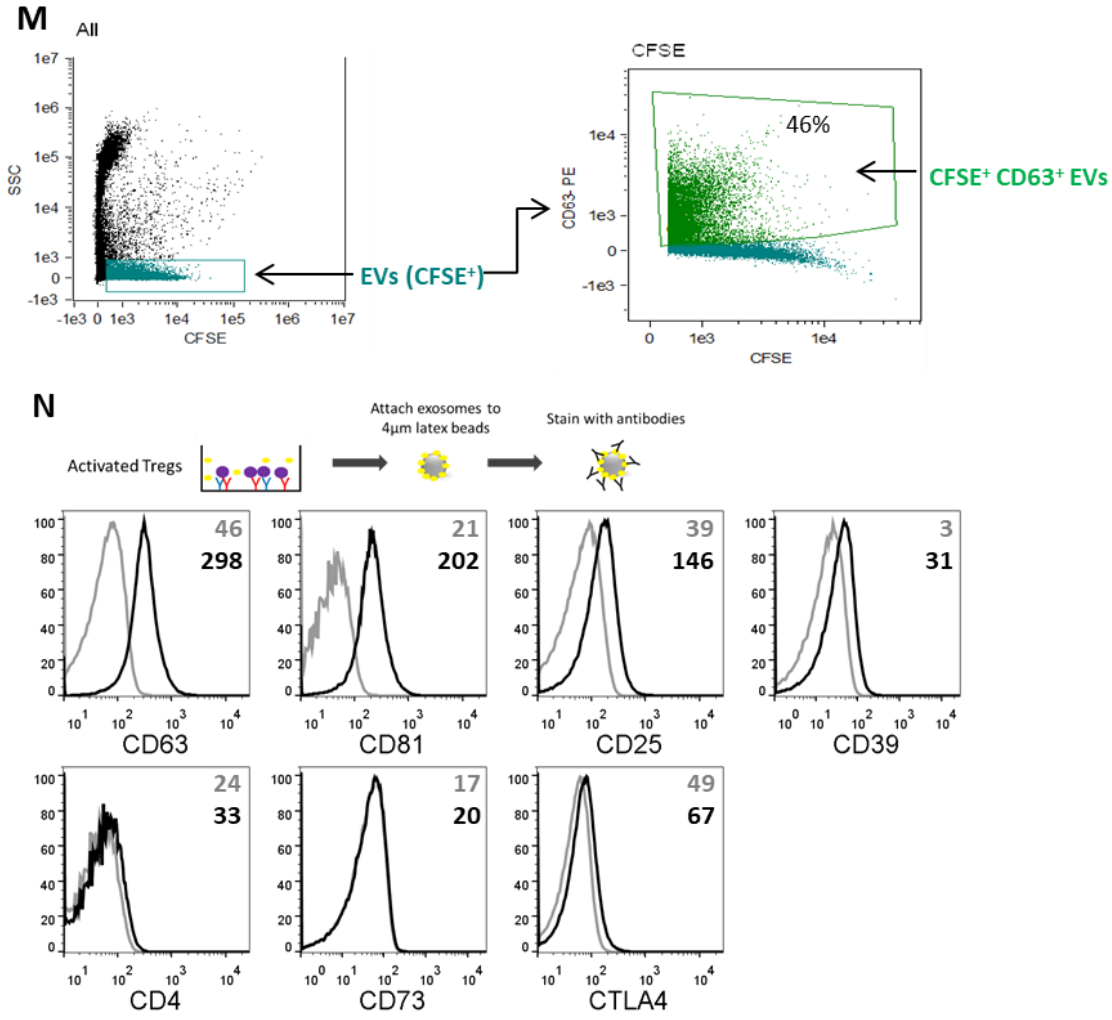


Figure 4.4- Activated Human Tregs and Teffs release EVs

Flow cytometry histogram plots showing the expression of CD69 in ***(A)*** *resting human Tregs (dashed line) and activated human Tregs (solid line) compared to isotype control (filled gray) or* ***(B)*** *Teffs. Values indicate mean fluorescence intensity (MFI) of isotype control (gray), resting cells (black) and activated cells (bold black) within each histogram plot. Data representative of 5 independent experiments. The y-axis indicates the percentage of maximum expression.* ***(C)*** *Transmission electron microscopy image of EVs released by activated human Tregs. Scale bar indicates 100nm. Representative of 3 independent experiments.* ***(D and E)*** *Representative NanoSight Nanoparticle Tracking Analysis (NTA) histogram showing the mean with red error bars indicating +/- 1 SEM. Bar graph shows pooled data for mode and mean particle size measurements of* ***(D)*** *Treg EVs from 12 individual experiments or* ***(E)*** *Teffs EVs from 9 individual experiments.* ***(F)***

Representative multiparameter graph showing particle intensity, diameter and concentration. Data representative of at least 21 individual experiments. Graphs showing the number of EVs released per cell from resting versus activated ***(G)*** *Tregs or* ***(H)*** *Teffs.* ***(I)*** *The numbers of EVs released per activated Treg and activated Teff cell were compared.* ***(J)*** *Tregs or* ***(K)*** *Teffs were activated for 0, 24 or 48 hours before enumerating the number of released EVs.* ***(L)*** *$CD63^+$ EVs detected and derived from equal numbers of Tregs or Teffs.* ***(M)*** *Image stream analysis plots showing the gating of EVs by positive CFSE staining and size of EVs. $CFSE^+$ EVs were further gated for $CD63^+$ expression. Representative of 2 independent experiments.* ***(N)*** *Flow cytometry histogram plots of Treg EVs bound onto latex beads showing the expression of CD63, CD81, CD25, CD39, CD4, CD73 and CTLA4 (black lines) compared to the control of stained latex beads alone (gray lines). Values indicate mean fluorescence intensity (MFI) of control stained latex beads (gray) and human Treg EVs bound onto latex beads (black). The y-axis indicates the percentage of maximum expression. Representative of 3 independent experiments.*

4.3.3 Human Tregs and Teffs suppress Tresps proliferation *in vitro*

Previous studies have shown that both Tregs and Teffs inhibit T cell proliferation (Trinchieri 2007; Lang, Wang, and Chen 2017; Putnam et al. 2013; Scottà et al. 2013). To confirm this with my expanded cells, CellTrace Violet™ (CTV)-labelled $CD4^+CD25^-$ Tresps were stimulated with anti-CD3/CD28 beads in the presence or absence of Tregs or Teffs at varying ratios of 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 of Tregs:Tresps or Teffs:Tresps.

As expected, Tresps stimulated with beads alone proliferated compared to Tresps cultured alone (**Figure 4.5-A**). At a 1:1 ratio of Tregs:Tresps, Tregs significantly suppressed Tresps proliferation, which on average was 88% inhibition compared to Tresps stimulated with beads alone ($p<0.0001$; **Figure 4.5-F**). The suppression was dose-dependent; the presence of Tregs at ratios of 1:2, 1:4, 1:8, 1:16 and 1:32 of Tregs:Tresps suppressed Tresps proliferation, an average at 84% ($p<0.0001$), 78% ($p<0.0001$), 68% ($p<0.0001$), 54% ($p=0.0003$) and 35% ($p=0.0005$) of suppression, respectively, compared to Tresps stimulated with beads alone (**Figures 4.5-B and F**).

At a 1:1 ratio of Teffs:Tresps, Teffs significantly suppressed Tresps proliferation, which was an average was 78% suppression compared to Tresps stimulated with beads alone

(p<0.0001; Figure 4.5-G). The suppression from Teffs was dose-dependent as well (Figures 4.5-C and G). The presence of Teffs at ratios of 1:2, 1:4, 1:8, 1:16 and 1:32 of Teffs:Tresps suppressed Tresps proliferation, an average at 71% (p<0.0001), 62% (p=0.0004), 49% (p=0.0034), 38% (p=0.0062) and 26% (p=0.0045) suppression, respectively, compared to Tresps stimulated with beads alone (Figures 4.5-C and G).

Overall, both Tregs and Teffs significantly inhibited Tresps proliferation which was in a dose dependent manner. Additionally, Tregs had a trend to inhibit Tresps proliferation at a higher level than Teffs.

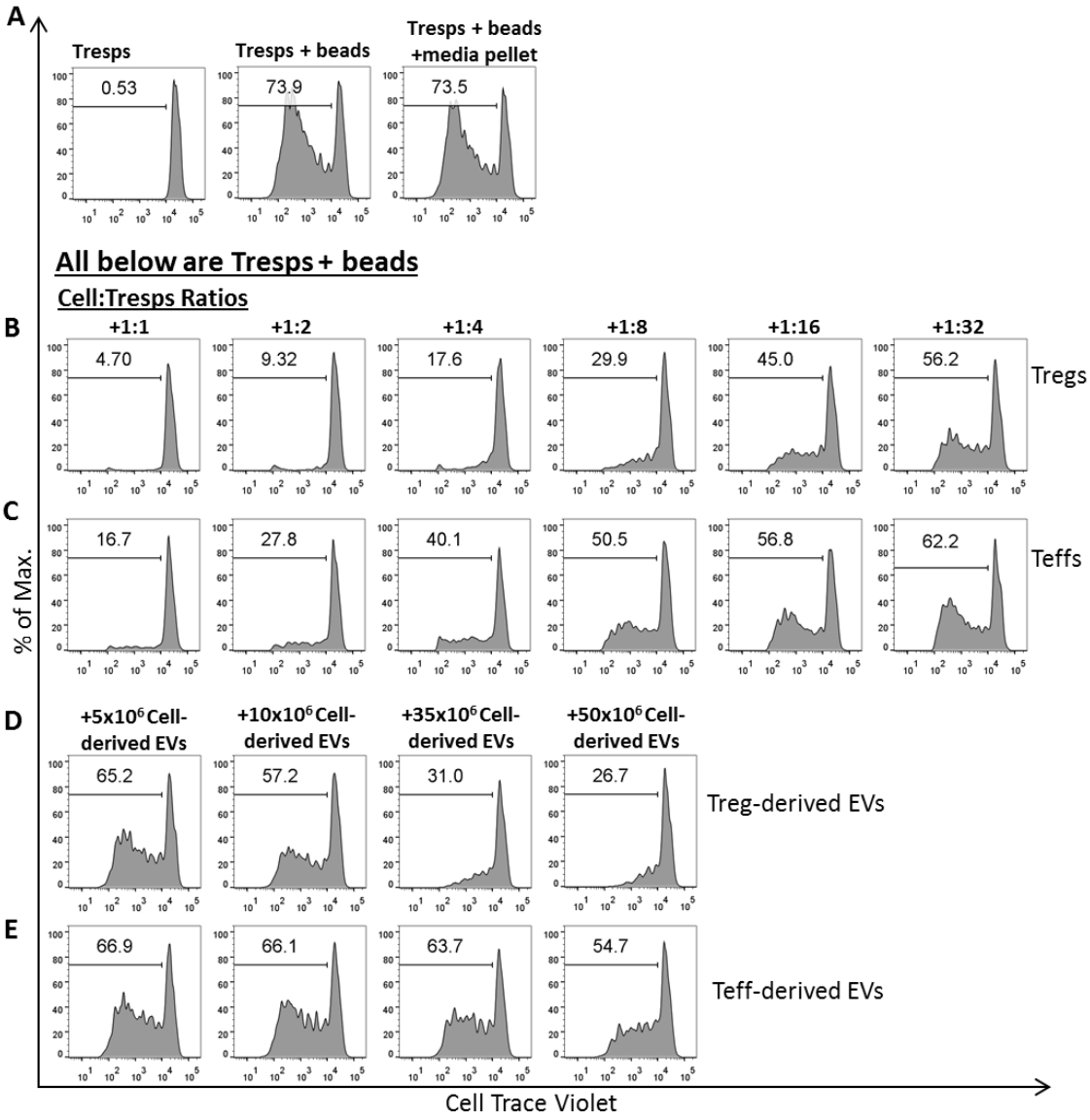
4.3.4 Human Treg EVs and Teff EVs suppress Tresps proliferation *in vitro*

Next, I investigated the efficiency of EVs derived from human Tregs to inhibit Tresps proliferation compared to EVs derived from human Teffs. To this end, CTV-labelled Tresps were stimulated with anti-CD3/CD28 beads in the absence or presence of Treg EVs or Teff EVs at varying quantities; 50, 35, 10 and 5 x10^6 Tregs derived EVs or Teffs derived EVs. In the presence of EVs derived from 50x10^6 Tregs significant suppression of Tresps proliferation compared to Tresps stimulated with beads alone was observed (p= 0.0384; Figures 4.5-A, D and H) which on average was approximately 35% suppression, with the highest suppression observed at 69%. This suppression from Treg EVs was dose-dependent (Figures 4.5-D and H), as the decreased amount of Treg EVs present, a reduced suppression was observed; the presence of 35, 10 and 5 x10^6 Tregs derived EVs suppressed Tresps proliferation by an average of 23%, 9% and 5% respectively.

The presence of Teff EVs derived from 50x10^6 Teffs also significantly suppressed Tresps proliferation compared to Tresps stimulated with beads alone (p= 0.0024; Figures 4.5-A, E and H) which on average was 22% suppression, with the highest suppression observed at 37%. This suppression from Teff EVs was also dose-dependent (Figures 4.5-E and H), the presence of 35, 10 and 5 x10^6 Teff derived EVs suppressed Tresps proliferation by an average of 18%, 10% and 8% respectively.

To test whether anything pelleted from the media using ExoQuick-TC™ was responsible for causing the suppression observed, the pellet resulting from the media alone was added to the Tresps stimulated with beads alone. No inhibition on the Tresps proliferation response was observed (Figures 4.5-A and I). Overall, both Treg EVs and

Teff EVs had immunomodulatory effects leading to significantly lower Tresps proliferation *in vitro*. However, no significant difference was observed between the suppressive capacity of Treg EVs and Teff EVs (Figure 4.5-H).



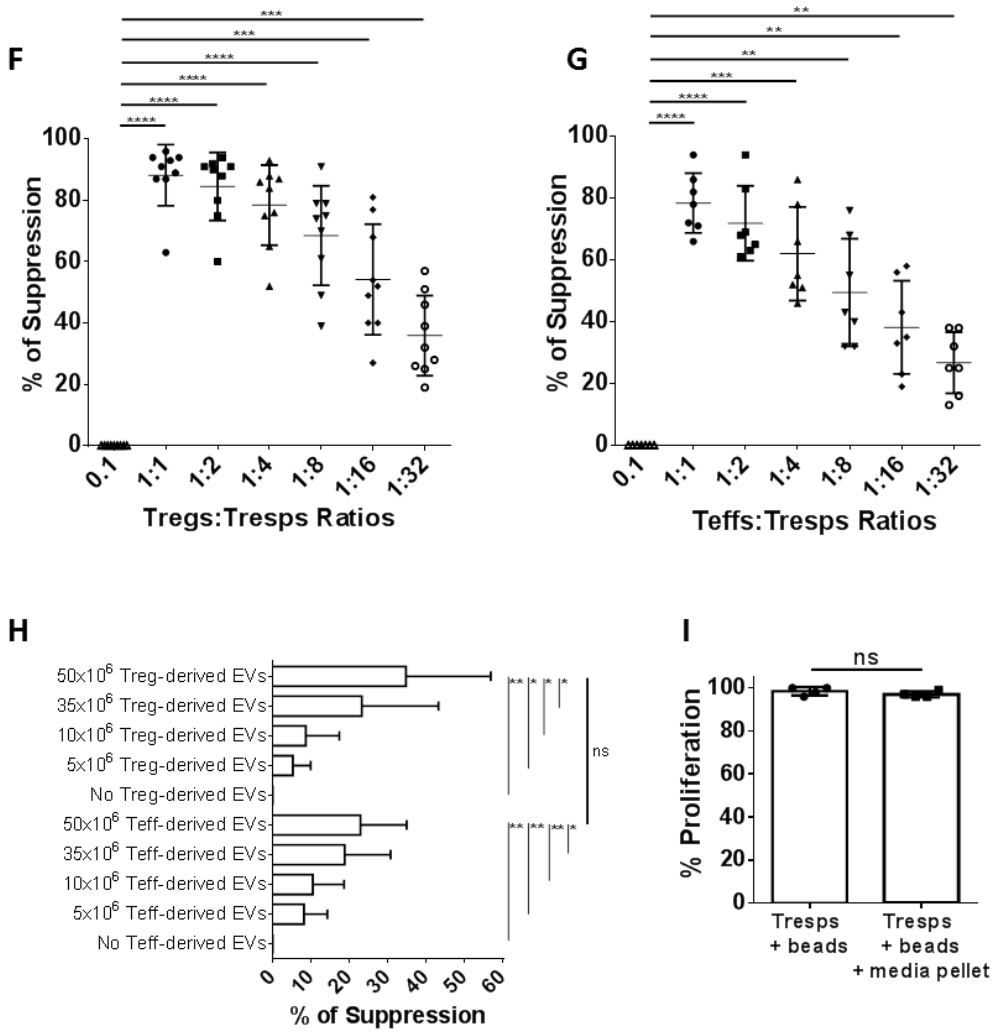


Figure 4.5- Human Treg EVs inhibit the proliferation of Tresps

Suppression assays results showing flow cytometry histogram plots of Tresps stained with CellTrace Violet™ to measure proliferation. ***(A)*** *Tresps were rested (unstimulated) or activated with anti-CD3/28 beads for 5 days, with the addition of media alone pellet. The addition of* ***(B)*** *Tregs or* ***(C)*** *Teffs were added to Tresps+ beads at the indicated ratios. The addition of* ***(D)*** *Treg EVs or* ***(E)*** *Teff EVs derived from the indicated number of cells were added to Tresps+ beads.* ***(A-E)*** *The y-axis indicates the percentage of maximum expression.* ***(F to I)*** *Graphs showing the pooled data of the dose-dependent suppression of Tresps proliferation (Tresps+ beads) by* ***(F)*** *Tregs* ***(G)*** *Teffs* ***(H)*** *Treg EVs and Teff EVs and* ***(I)*** *media pellet. Data are expressed as percentage of suppression of Tresps proliferation relative to Tresps cultured with anti-CD3/28 beads alone. Graphs represent pooled data from at least 7-9 independent experiments, except media pellet data show n=4. Bars show mean + SD. Statistical significance was tested using one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= non-significant.*

4.3.5 Human Treg EVs are acquired by Tresps

I have shown in section 3.3.6 that mouse Treg EVs are acquired by BM-DCs. Torri *et al.* have previously shown that human Treg EVs are taken up by HuH-7 cancer cells (Torri et al. 2017). However, they did not assess whether human Treg EVs are acquired by T cells like their mouse counterparts (Okoye et al. 2014). In order to investigate whether human Treg EVs are acquired by Tresps, Tregs were labelled with CFDA-CFSE and activated; the secreted $CFSE^+$ EVs were isolated and co-cultured with Tresps for 24 hours before washing extensively. Tresps were acquired on the flow cytometer to assess their CFSE status. $CFSE^+$ Treg EVs had acquired the $CFSE^+$ dye. The FACS plot indicated that Tresps that were co-cultured with $CFSE^+$ Treg EVs had a higher CFSE MFI compared to Tresps cultured alone (MFI of 599 versus 350, respectively; Figure 4.6), indicating that $CFSE^+$ Treg EVs had been acquired by Tresps. Due to time limits on the PhD project, the co-culture of $CFSE^+$ Teffs EVs with Tresps was not performed. The results presented here indicated that *in vitro* Tresps acquire Treg EVs.

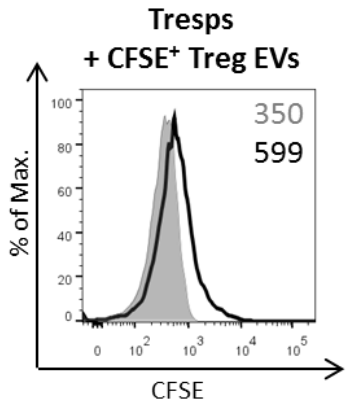


Figure 4.6- Human Treg EVs are acquired by Tresps

Flow cytometry histogram plot showing Tresps alone (gray filled) or Tresps co-cultured with $CFSE^+$ Treg EVs for 24 hours. After 24 hours, Tresps were washed twice prior to acquisition on the flow cytometry. Values indicate mean fluorescence intensity (MFI) of Tresps alone (gray) and Tresps co-cultured with $CFSE^+$ Treg EVs (black).

4.3.6 Human Tregs and Teffs modulate Tresps cytokine production profiles

Previous reports have shown that human Tregs can modulate cytokine production of T cells by inhibiting the production of pro-inflammatory cytokines such as IL-2 and IFNγ (Sojka, Huang, and Fowell 2008; Hoeppli et al. 2015; S. Sakaguchi et al. 2009). Teffs also modulate the cytokine production of T cells (W. G. H. Han et al. 2010; Pawelec et al. 1996; Fagiolo and Toriani-Terenzi 2002). To confirm these findings with my cells, the supernatants taken from the suppression assays were tested using Cytometric Bead Array (CBA) kits.

Tresps stimulated with anti-CD3/CD28 beads produced high levels of IFNγ, however the presence of Tregs at 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 ratios significantly decreased IFNγ production levels in a dose dependent manner (all $p<0.0001$) (**Figure 4.7-A**). Pro-inflammatory cytokine IL-2, which is produced by Tresps were also significantly reduced in the presence of Tregs at 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 ratios (all $p<0.0001$; **Figure 4.7-B**). IL-6 and TNF, were also produced by Tresps. Like the aforementioned cytokines, the levels of IL-6 and TNF released by Tresps was also significantly reduced in the presence of Tregs at 1:1, 1:2, 1:4, 1:8 and 1:16 ratios (IL-6; $p<0.0001$, $p=0.0002$, $p=0.0006$, $p=0.0012$ and $p=0.0194$, respectively; and TNF; $p<0.0001$, $p<0.0001$, $p<0.0001$, $p=0.0002$, $p=0.0006$ and for 1:32 ratio $p=0.0028$; **Figure 4.7-C and F**).

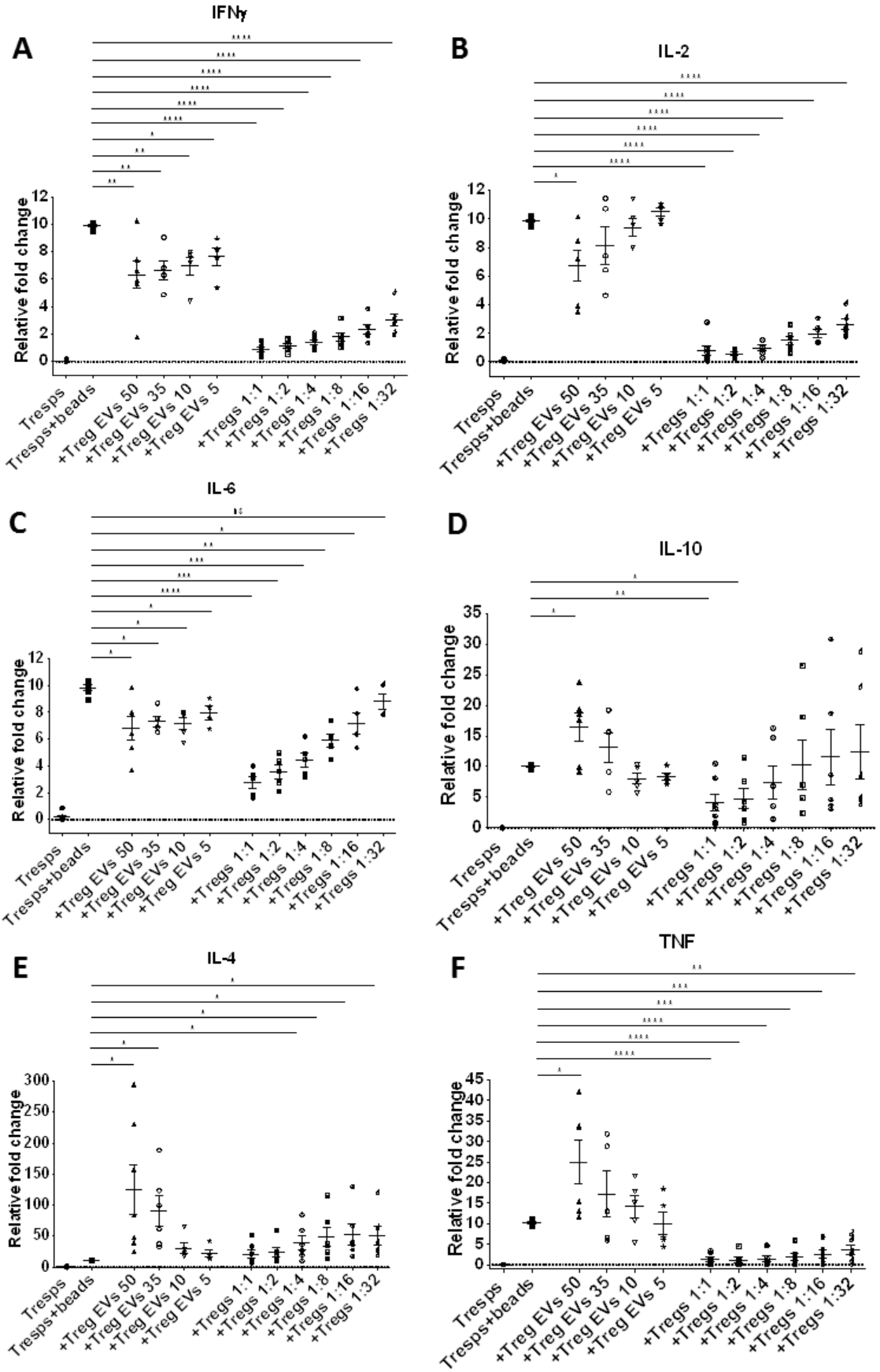
IL-10 was significantly decreased in the presence of Tregs at the ratios of 1:1 and 1:2 ($p=0.0053$ and $p=0.0237$, respectively) but had a trend to increase IL-10 production levels at the other cell ratios tested; 1:4, 1:8, 1:16 and 1:32 (**Figure 4.7-D**). IL-4 production was significantly increased in the presence of Tregs at a 1:4, 1:8, 1:16 and 1:32 ratios ($p=0.0473$, $p=0.0497$, $p=0.0495$, $p=0.0444$, respectively) (**Figure 4.7-E**).

Despite Teffs having the capacity to inhibit Tresps proliferation (**Figures 4.5-C and G**), they functioned differently to Tregs, in regard to affecting cytokine production by Tresps. Namely, unlike Tregs, Teffs had a trend to increase the production levels of IFNγ, although this did not reach statistical significance (**Figure 4.7-G**). Nonetheless, as expected the IL-2 production levels were decreased by the addition of Teffs at the 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 ratios ($p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.0001$, $p<0.0001$ and $p=0.0001$, respectively) (**Figure 4.7-H**). In the presence of Teffs, there was an increase of IL-6 production levels, at 1:4 and 1:16 ratios ($p=0.0304$ and $p=0.0475$,

respectively) and an increase of IL-10 prolduction levels, at 1:4, 1:8, 1:16 and 1:32 ratios (p=0.0337, p=0.0306, p=0.0269 and p=0.0304, respectively) was observed (**Figure 4.7-I and J**). Lastly, Teffs did not significantly alter the production levels of IL-4 (**Figure 4.7-K**) or TNF (**Figure 4.7-L**) by Tresps.

Taken together, this demonstrated that human Tregs have the capacity to significantly inhibit pro-inflammatory IFNγ, IL-2, IL-6 and TNF cytokine production levels by Tresps whilst increasing their IL-4 and IL-10 cytokine production levels.

Notably, human Teffs acted differently to Tregs, as Teffs promoted rather than inhibited the production levels of IFNγ and significantly increased IL-6 production levels by Tresps. However, human Teffs inhibited IL-2 cytokine production levels whilst also increasing IL-10 production levels. Furthermore, IL-4 and TNF production levels by Tresps were generally unaltered by the presence of Teffs.


A
IFNγ
Relative fold change
B
IL-2
Relative fold change
C
IL-6
Relative fold change
D
IL-10
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E
IL-4
Relative fold change
F
TNF
Relative fold change
Tresps
Tresps+beads
+Treg EVs 50
+Treg EVs 35
+Treg EVs 10
+Treg EVs 5
+Tregs 1:1
+Tregs 1:2
+Tregs 1:4
+Tregs 1:8
+Tregs 1:16
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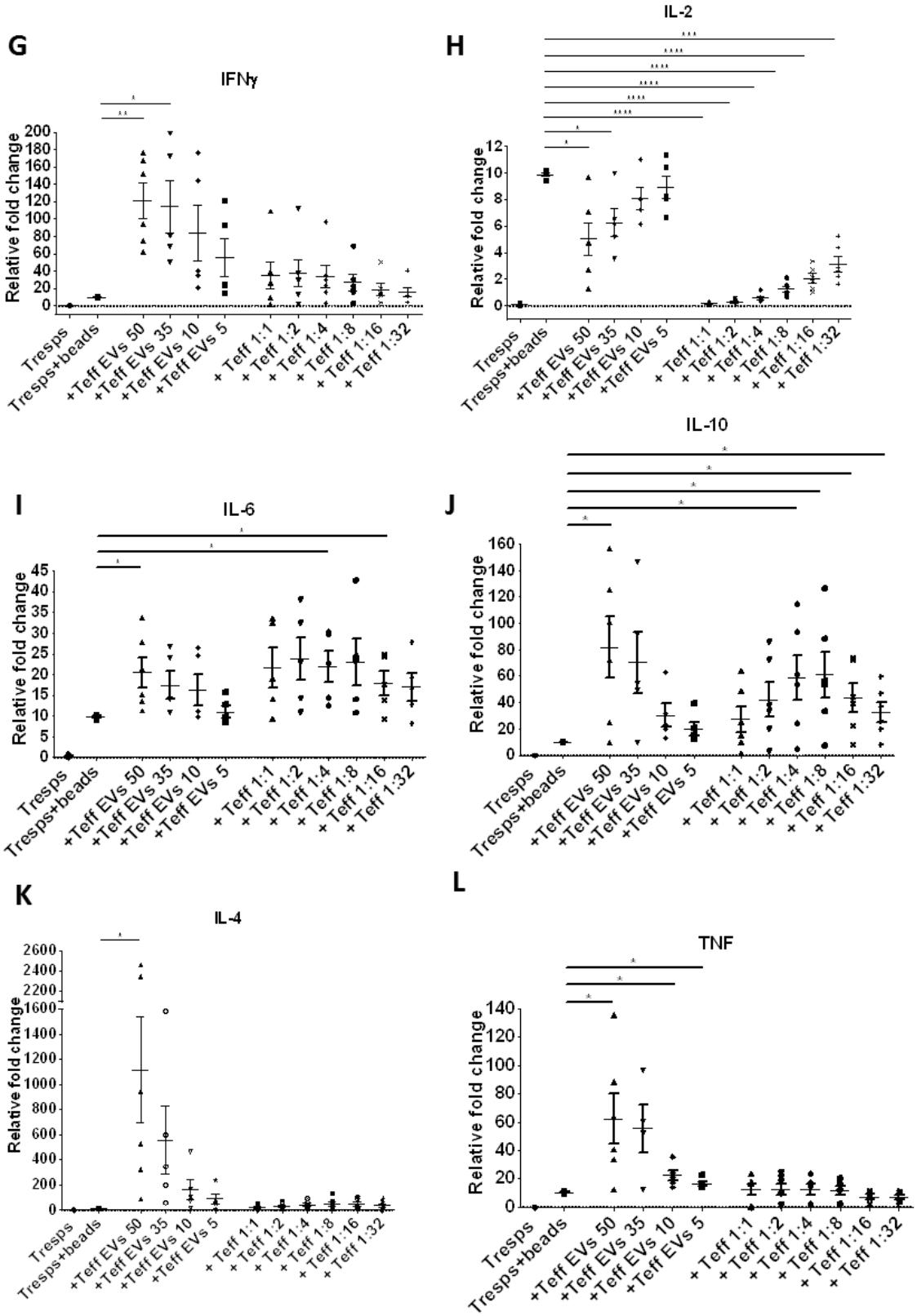


Figure 4.7- Human Tregs, Treg EVs, Teffs and Teff EVs modulate cytokine production profiles of Tresps

Graphs show the relative fold change of cytokine levels detected in the supernatant of the co-cultures of Tresps with the various indicated amounts of ***(A-F)*** *Treg EVs or Tregs*

or **(G-L)** *Teff EVs or Teffs. Cytokines of IFNγ, IL-2, IL-6, IL-10, IL-4 and TNF were assessed. Treg EV no. or Teff EV no. represents no. x 10^6 Treg-derived EVs or Teff-derived EVs. Bars show mean ± SD. Data represents pooled data from at least 5 independent experiments. Each assay was performed in technical triplicates. The value from each assay of Tresps+ beads was set at a value of 10 fold and the relative quantities from each condition is expressed relative to Tresps+ beads. Statistical significance was tested using one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= non-significant.*

4.3.7 Human Treg EVs and Teff EVs modulate Tresps cytokine production profiles

Having confirmed that human Tregs and Teffs modulate cytokine production by activated Tresps, I analysed whether EVs released by these cells affected cytokine production in a similar way. Previous reports have shown that mouse Treg EVs can affect cytokine production of the T cells (Lesley Ann Smyth et al. 2013; Okoye et al. 2014) and BM-DCs (**Chapter 3**), suggesting that human Treg EVs may alter the cytokine production by Tresps. In order to test this, supernatant of the co-cultures from the suppression assays (**Figure 4.5**) were tested for the presence of the various cytokines outlined above.

As expected, Tresps stimulated with anti-CD3/CD28 beads produced high levels of IFNγ, but the presence of 50, 35, 10 and 5 x10^6 Treg derived EVs significantly reduced IFNγ production levels (p=0.0076, p=0.0056, p=0.006 and p=0.0124, respectively) (**Figure 4.7-A**) and responded according to a dose dependent manner (**Figure 4.7-A**).

Similarly, IL-2 was also significantly reduced in the presence of 50x10^6 Treg derived EVs (p=0.034) (**Figure 4.7-B**). IL-6 was also significantly reduced in the presence of 50, 35, 10 and 5 x10^6 Treg derived EVs (p=0.0311, p=0.0114, p=0.0147 and p=0.0106, respectively) (**Figure 4.7-C**). Interestingly, not only does 50x10^6 Treg derived EVs inhibit cytokine release but simultaneously they appeared to significantly increase the production levels of IL-10 by Tresps (p=0.0449) (**Figure 4.7-D**). The pleiotropic cytokine IL-4 was significantly increased by 50 and 35 x10^6 Treg derived EVs (p=0.0268 and p=0.0215, respectively) (**Figure 4.7-E**). 50x10^6 Treg derived EVs increased the TNF production by Tresps (p=0.0414) (**Figure 4.7-F**).

On the contrary, Teff EVs functioned differently to Treg EVs in regard to affecting cytokine production by Tresps. Namely unlike Treg EVs, Teff EVs derived from 50 and 35 $x10^6$ Teffs significantly increased the production levels of IFNγ (p= 0.0028 and p=0.0250, respectively) (**Figure 4.7-G**). The IL-2 production levels were decreased by 50 and 35 $x10^6$ Teff derived EVs (p= 0.0111 and p=0.0248, respectively) (**Figure 4.7-H**). IL-6 was significantly increased by the presence of $50x10^6$ Teff derived EVs (p= 0.029) (**Figure 4.7-I**). Interesting, $50x10^6$ Teff derived EVs significantly increased the production levels of IL-10 (p= 0.0274) (**Figure 4.7-J**) and IL-4 (p= 0.0478) (**Figure 4.7-K**). TNF production levels were increased by 50, 10 and 5 $x10^6$ Teff derived EVs (p= 0.0341, p=0.0306 and p=0.0143, respectively) (**Figure 4.7-L**).

Overall, these results demonstrate that human Treg EVs have the capacity to significantly inhibit pro-inflammatory IFNγ, IL-2 and IL-6 cytokine production by Tresps whilst increasing their IL-10 and IL-4 cytokine production levels, perhaps towards creating an anti-inflammatory microenvironment. Furthermore, Treg EVs also increased TNF production by Tresps. Human Teff EVs acted differently to Treg EVs, as Teff EVs promoted rather than inhibited the production levels of IFNγ and IL-6 by Tresps. However, human Teff EVs also inhibited IL-2 cytokine production levels whilst also increasing IL-10, IL-4 and TNF production levels by Tresps, which is in a similar manner to Treg EVs.

4.4 Discussion

4.4.1 Phenotype of Tregs and Teffs

The phenotype profile of human Tregs and Teffs were similar but Tregs had a distinct higher percentage of cells expressing CD25, FoxP3 and CTLA4 compared to Teffs, in line with previous studies (Kmieciak et al. 2009; Jago et al. 2004; Jun Wang et al. 2007; Mason et al. 2015; Finney, Riley, and Walther 2009). Despite their overlapping phenotype expression profiles, human Tregs and Teffs are functionally divergent. However, currently, there is not a single exclusive marker to distinguish between human Tregs from Teffs, but rather a combination of cell surface and intracellular markers and their differing expression levels are required to discriminate them. As explained in **section 1.6.2**, human $CD4^+$ Tregs are $CD25^{high}$ and $FoxP3^+$. On the contrary, human Teffs

are $CD25^-$ and $FoxP3^{low/-}$ but as demonstrated by Kmieciak *et al*., human Teffs transiently upregulate CD25 and FoxP3 post-activation (Kmieciak et al. 2009). Due to this, challenges exist to separate the two cell populations. Hence, the observation that Teffs expressed CD25 and FoxP3, albeit to a lower level than Tregs is therefore not surprising.

Previous reports demonstrate that Tregs express surface CD63 (J. Zheng et al. 2013) whilst T cells express surface and intracellular CD63 (Pfistershammer et al. 2004), however CD63 cell surface expression of resting T cells are low but increase upon activation (Pfistershammer et al. 2004). Pfisterhammer *et al*. demonstrated that CD63 is an activation linked T cell costimulatory element which induced their proliferation and their production levels of IL-2 (Pfistershammer et al. 2004). The role of cellular CD63 was not tested in this project but may have contributed to the cellular functions of Tregs and Teffs.

4.4.2 Suppressive ability and cytokine modulation of Tregs and Teffs towards Tresps

I demonstrated that human Tregs and Teffs inhibit Tresps proliferation; their suppressive ability was in a dose dependent manner. As described in section 1.6.3, Tregs can use a number of different molecular mechanisms to suppress their target cell functions.

Human Tregs and Teffs modulate the cytokine production by Tresps. Namely, Tregs are known to suppress IFNγ and IL-2 whilst increasing IL-10 production levels by T cells to create an anti-inflammatory microenvironment. I have shown that both human Tregs and Teffs modify Tresps cytokine production profile. Notably, human Teffs responded differently to Tregs, as Teffs had a trend to promote rather than inhibit the production of IFNγ and also significantly increase IL-6 production by Tresps. However, human Teffs inhibited IL-2 cytokine production whilst simultaneously increasing IL-10. In addition, Teffs did not affect the IL-4 and TNF production levels by Tresps.

4.4.3 Release of EV numbers from activated Tregs and Teffs

Both human Tregs and Teffs released EVs upon TCR activation, in line with published data (Torri et al. 2017). Noteworthy, human Teffs released significantly more EVs than Tregs, although Tregs released more $CD63^+$ EVs than Teffs. This is in agreement with the mouse setting, whereby Okoye *et al*. demonstrated that mouse Tregs released more $CD63^+$ EVs in comparison to $CD4^+$ naïve T cells, $CD8^+$ naïve T cells, B cells, Th1 or Th17 cells (Okoye et al. 2014). Although, cell activation induced a significant increase of secreted EVs from human Tregs, the secretion of EV numbers continued to elevate from 24 to 48 hours post-activation. Despite the depletion of activation beads 2 days prior to isolating EVs, it cannot be ascertained that these cells were truly 'rested' which may explain the numbers of EVs detected at their 'resting Treg' status. Nevertheless, these human Treg EVs were collected 24 hours post-activation, which is in contrast to Torri *et al.* who collected human Treg EVs at 72 hours post-activation (Torri et al. 2017). As explained above, to avoid collection of apoptotic bodies, EVs released at 24 hours were used instead of 48 hours derived EVs.

4.4.4 Treg EVs are acquired by Tresps

I have shown that human Treg EVs were acquired by Tresps directly. How EVs interact with target cells is not completely understood but has been suggested to involve various clathrin-dependent and independent endocytosis, phagocytosis, micropinocytosis, receptor-ligand mediated pathways, cell surface membrane fusion and protein interactions (Mulcahy, Pink, and Carter 2014; Maas, Breakefield, and Weaver 2017; Costa Verdera et al. 2017). This interaction was demonstrated to functionally affect Tresps by modulating their proliferation and cytokine production profiles. Whether EVs use receptor mediated uptake by target cells has been explored (Mulcahy, Pink, and Carter 2014; Maas, Breakefield, and Weaver 2017; Costa Verdera et al. 2017). Proteomics and mass spectrometry has been used by various other groups to reveal the contents of EVs (Haraszti et al. 2016; Pietrowska et al. 2017; C. Yang et al. 2017; Pocsfalvi et al. 2016; Joy et al. 2018). As such, for this study, a similar approach can be employed to further understand Treg EV receptor and protein contents.

4.4.5 Suppressive ability of Treg EVs and Teff EVs

APC derived EVs can be 10-20 times less efficient at stimulating T cell responses compared to the parent APC (Zitvogel et al. 1998; Vincent-Schneider et al. 2002). However, the response can depend on other factors such as how many EVs were added to the target cells and therefore how many active molecules were transferred into the target cells to observe a detectable response. Hence, due to these factors, I used multiple doses of Treg EVs and Teff EVs to observe a dose dependent response. I showed that Treg EVs and Teff EVs suppress Tresps proliferation which the suppressive response was at the most potent at $50x10^6$ Treg derived EVs or Teff derived EVs. However, of note the suppression observed could be due to EVs actively suppressing Tresps or these EVs could be binding to CD3/CD28 beads and thus interfere or block Tresps activation leading to their reduced proliferation.

Despite that EVs were derived from the same number of starting parent cells and given that Teffs released approximately 2.4 fold more EVs than Tregs, if based on these EV numbers, the Treg EVs suppressed Tresps proliferation more efficiently than Teff EVs. However, Torri *et al.* also further stated that Treg EVs but not Th1 EVs nor Th17 EVs inhibited $CD4^+$ T cell proliferation (Torri et al. 2017). This is in contrast with my findings that human Teff EVs are immunosuppressive. As described in section 1.7.7, Teff EVs can be both anti-inflammatory and pro-inflammatory. Torri *et al*. used EVs derived from 72 hours post-cell activation (Torri et al. 2017), whether these EVs contained apoptotic bodies is not clear but given the heterogeneity in EVs and thus the active biological components in EVs could be different and especially as the methods used to isolate EVs could result in the differences between Torri *et al.'s* finding that neither Th1 EVs nor Th17 EVs inhibited T cell proliferation (Torri et al. 2017), whereas, herein I observed that Teff EVs inhibited Tresps proliferation. Torri *et al.* used ExoMir™ Mini kit, Exo-spin™ kit and ultracentrifugation methods of EV isolation (Torri et al. 2017), whereas I used ExoQuick-TC™, thus, it is likely that different EV populations may have been isolated and hence may provide an explanation for the different results.

4.4.6 Th1 and Th17 EVs- Torri *et al*.'s publication

On closer inspection of Torri *et al*.'s publication, the data to demonstrate Th1 EVs ability to inhibit $CD4^+$ T cell proliferation was missing (Torri et al. 2017). Furthermore, Treg EVs and Th17 EVs were compared for immunosuppression ability via three different approaches: (1) Percentage of cell division (2) Division Index and (3) Proliferation Index. Although the authors do state that the percentage of cell division was clearly not different with Treg EVs or Th17 EVs treatment, they focused on the division and proliferation index values. The division index is an algorithm that calculates the average number of cell divisions that a cell from the original population has undergone which also takes into account the undivided cells (Division index= Total number of divisions ÷ Total number of cells at start of culture). Whereas, the proliferation index is an algorithm that calculates only the cells that have undergone at least one division and thus does not take into account the undivided cells (Proliferation index= Total number of divisions ÷ Number of cells that underwent division). Torri *et al*. showed that Th17 EVs decreased the T cell division index, which can be interpreted as Th17 EVs are, in fact, immunosuppressive. However, on the other hand, because the proliferation index was increased above the control, it was interpreted as non-immunosuppressive. This discrepancy in the interpretation of the results was not clearly stated by the authors (Torri et al. 2017) and questions whether Th17 EVs are in fact immunosuppressive.

4.4.7 Phenotype of Treg EVs

The Treg $CD63^+$ $CD81^+$ EVs while they contained Treg cell-associated markers such as CD25 and CD39, very low or negligible levels of CD4 was observed on their vesicular surfaces. Given that the percentage of parent Tregs exhibits over 97% and 91% of expression levels of both CD4 and CD25, respectively, but only CD25 was found on EVs further supports the concept that EVs are not a mere smaller version of their parent cell but rather an EV that has cargo selected and packaged in a specific manner (Clotilde Théry, Zitvogel, and Amigorena 2002).

Molecules on the surface of EVs are functionally used by EVs to conduct their immunomodulation. For example, a study by Clayton *et al*. in 2011 showed that cancer exosomes contain CD39 and CD73 ectoenzymes on their surface, which suppressed T

cells activity by converting the pro-inflammatory ATP into anti-inflammatory adenosine (A. Clayton et al. 2011). Similarly, my host laboratory has also previously shown the presence of CD73 on EVs derived from mouse Tregs which contributed to their immunosuppressive ability towards proliferating T cells. Furthermore, these murine Tregs EVs caused a reduction of IL-2 and IFNy production by target cells *in vitro*, leading to an immunosuppressed microenvironment (Lesley Ann Smyth et al. 2013). Given that both CD25 and CD39 are immunosuppressive molecules used by the Treg cell themselves (Vignali, Collison, and Workman 2008; Borsellino et al. 2007; Shimon Sakaguchi, Wing, and Miyara 2007), it is tempting to suggest that these molecules found on our Treg EVs could also be involved in inhibiting the immune response.

Given that the host laboratory demonstrated that mouse Treg EVs express CD73 which directly contributed to their suppressive function (Lesley Ann Smyth et al. 2013), it highlighted a similar possibility with human Treg EVs. I did not find CD73 enriched in human Treg EVs but the presence of CD39 molecules on human Treg EVs was observed. Previous studies by others have shown that T cells express A2A adenosine receptors (Ohta et al. 2009; Koshiba et al. 1999; Linden and Cekic 2012), suggesting that this CD39 ectoenzyme on Treg EVs could potentially explain in part their mode of immune suppression observed in our co-cultures with Tresps. In the mouse Treg setting, CD39 is co-expressed with CD73 (Deaglio et al. 2007; Lesley Ann Smyth et al. 2013). On the contrary, human Tregs can independently express CD39 and CD73 (Dwyer et al. 2010; Mason et al. 2015). Furthermore, the human $CD39^{hi}$ Treg subset is more stable and functionally more suppressive that the $CD39^{low}$ Treg counterparts (Gu et al. 2017). The human Tregs used in this chapter had a higher expression level of CD39 compared to CD73 and given that human $CD39^{hi}$ Tregs are more suppressive than human $CD39^{low}$ Tregs (Gu et al. 2017), the expression of CD39 on human Treg EVs may be a possible mechanism used by Treg cells to increase their suppressive ability. CD25 is a high affinity receptor for IL-2; Tregs express high levels of this molecule which has been shown to deplete IL-2 survival cytokine from the microenvironment, thus preventing Teffs proliferation (S. Sakaguchi et al. 2009; Corthay 2009). Nolte-'t Hoen *et al.* published that MHC-II/peptide complexes-bearing vesicles derived from murine anergic T cells can be taken up by APCs and endow them with immune-suppressive properties. These vesicles also contained CD80, CD86, CD2, CD54 and CD25 which may have played

a role in their immunosuppressive ability (Nolte-'t Hoen et al. 2004). Thus, the $CD25^+$ exosomes bound to the cell surface of APCs, providing the APCs with the ability to deplete IL-2 from the microenvironment leading to apoptosis of Teffs (Nolte-'t Hoen et al. 2004).

I have shown that human Tregs express high levels of both CD4 and CD25 but only CD25 and negligible levels of CD4 were found on their EVs. Whether CD25 was a mechanism used by human Treg EVs to deplete IL-2 from the microenvironment was not tested but given that Tresps cultured with Treg EVs had reduced IL-2 levels, points to this possibility. Why CD4 was not packaged into EVs remains to be determined but other previous reports also described that EVs did not contain markers that are highly expressed by their parent cells. For example, T cell derived exosomes did not display the abundant parent cell-surface CD45 and CD28 but did however contain TCR/CD3ζ complexes (Blanchard et al. 2002). Moreover, Thery *et al*. demonstrated that DCs which express high levels of Fc receptors produced exosomes which lacked these cell surface molecules (C Théry et al. 1999), further supporting a regulated packaging mechanism of EVs.

4.4.8 Cytokine modulation of Treg EVs and Teff EVs

Although the treatment of Tresps with Treg EVs did inhibit IL-2 production, they only significantly reduced this at the highest Treg EV dose used ($50x10^6$ Treg derived EVs). Although Teff EVs were immunosuppressive as well, their mechanisms of suppression may be different to that of Treg EVs given their differential effects in modulating cytokine production by Tresps, thus these EVs were different in function and highlights the heterogeneity in EVs. The human Treg EVs significantly inhibited pro-inflammatory IFNγ and IL-6 cytokine production levels by Tresps, whereas Teff EVs promoted rather than inhibited their production levels of IFNγ and IL-6 cytokines. In addition, the Treg EVs also increased TNF production levels by Tresps. However, for other cytokines human Teff EVs acted similar to Treg EVs in that they also inhibited IL-2 cytokine production whilst also increasing IL-10, IL-4 and TNF production levels by Tresps. This modulation of cytokine milieu by Treg EVs may be a mechanism to create an anti-inflammatory microenvironment. The diverse cytokine modulation roles of Treg EVs and

Teff EVs could be due to their miRNA content, as suggested for mouse Treg EVs (Chapter 3) and this will be addressed in the next chapter (Chapter 5).

4.4.9 Tregs and Treg EVs differences and similarities

I have demonstrated that Tregs and their EVs have some similar roles but also EVs acted differently *in vitro*. I demonstrated that human Treg EVs, akin to their parent cells, although not as potently can inhibit Tresps production levels of pro-inflammatory IFNγ, IL-2 and IL-6 cytokines (Tiemessen et al. 2007; Sojka, Huang, and Fowell 2008; Hoeppli et al. 2015; S. Sakaguchi et al. 2009). Concurrently, Treg EVs treatment *in vitro* increased Tresps production levels of immunosuppressive cytokines; IL-10 and IL-4. Parent Tregs increased IL-4 production by Tresps in line with previous studies (Tiemessen et al. 2007), however, for some Treg donors it promoted IL-10 production whereas other Treg donors inhibited IL-10 release by Tresps, this may be due to human variability. However, TNF production by Tresps was inhibited by Tregs, in contrast, Treg EVs notably increased TNF release. But as shown in the next chapter (Chapter 5) Treg EVs have exclusive miRNAs not found in the parent Tregs which may explain their differential role in modulating TNF cytokine production.

4.4.10 Teffs and Teff EVs differences and similarities

Compared to Treg EVs, Teff EVs did not suppress the production of IFNγ by Tresps, but instead significantly increased their production levels, in a manner that reflected the trend of parent Teffs towards Tresps, although this did not reach statistical significance. Teffs EVs akin to their parent Teffs and akin to Tregs and Treg EVs inhibited the production levels of IL-2. Teff EVs and their parent Teffs responded differently compared to Treg EVs and Tregs by increasing the production levels of IL-6 by Tresps. This finding highlighted that Treg EVs and Teff EVs although both are immunosuppressive; they have different effects pertaining to cytokine release. Teff EVs also induced the production levels of IL-10, IL-4 and TNF by Tresps. This is further discussed below.

4.4.11 Cytokine modulation by Tregs, Teffs, Treg EVs, and Teff EVs in relation to literature findings

The differentiation of precursor Th cells into Th1 or Th2 subsets is highly influenced by the presence and levels of IL-4 or IFNγ within the microenvironment during differentiation (Paludan 1998). Whilst IL-4 polarises Th2 differentiation and inhibits Th1 differentiation, IFNγ polarises Th1 differentiation and inhibits Th2 differentiation. Th2 immune deviation have previously been linked to transplant tolerance (Walsh, Strom, and Turka 2004), however this is a complex mechanism. Whilst some groups report that skewing of alloimmunity towards a Th2 phenotype by anatagonising IL-12 signalling or by *in vivo* injection of Th2 cytokines still exacerbated rejection of MHC mismatched grafts (X. C. Li et al. 1998; Piccotti et al. 1996; X. X. Zheng et al. 1995), but polarising a Th1 response towards a Th2 response is adequate to promote transplant survival across selected minor histocompatibility barriers (X. C. Li et al. 1998)

IL-4 cytokine is a critical factor involved in the suppression of the pro-inflammatory cytokine response (Tiemessen et al. 2007). Silverman *et al*. demonstrated that Leishmania exosomes-treated mice had a higher number of IL-4 producing $CD4^+$ T cells whilst reducing the number of IFNγ producing $CD4^+$ T cells (Silverman et al. 2010), highlighting that exosomes/EVs can modify $CD4^+$ T cells cytokine production levels *in vivo*. In the co-cultures, the Treg EVs decreased IFNγ production levels whilst increased the IL-4 production levels, taken with the above publications this finding suggests that Treg EVs may be promoting a polarisation towards a Th2 differentiation profile.

Given that EVs are carriers of cytokines (A. Clayton et al. 2007; Tokarz et al. 2015; Hasegawa et al. 2011; Szabó et al. 2014; Konadu et al. 2015) and EVs can offload their contents into target cells, it is conceivable that Treg EVs may have directly transferred IL-4 along with other cytokines into target cells rather than being directly upregulated in $CD4^+$ T cells, as human Tregs can produce IL-4 cytokine (Tiemessen et al. 2007). However, I have not tested the direct transfer of cytokines via EVs due to time limits on the PhD project.

Tregs can use a combination of suppressive mechanisms, but in some *in vitro* assays, it appeared to be independent of IL-10 and TGF-b (M K Levings, Sangregorio, and Roncarolo 2001; Taams et al. 2001; A M Thornton and Shevach 1998), whereas others

report a major role of IL-10 (Chaudhry et al. 2011; Collison et al. 2009). I observed a significant increase in IL-10 with Treg EVs treatment but only at the highest dose of Treg EVs. This suggests that Treg EVs rather than inducing a general cytokine modulation of production in Tresps; it affects specific cytokines, perhaps shifting the balance from a pro-inflammatory towards an anti-inflammatory cytokine microenvironment.

Tregs can suppress IL-6 production (Tiemessen et al. 2007), I have shown that human Treg EVs akin to their parent Tregs can also significantly inhibit IL-6 cytokine production. IL-6 has been shown by various groups to drive pro-inflammatory microenvironments and transplant rejection (Luqiu Chen et al. 2009; Zhao et al. 2012; Shen and Goldstein 2009) and is suggested to be involved in the Th1/Th2 balance (Zhao et al. 2012). Zhao *et al*. demonstrated that wild-type C57BL/6 mice which received CTLA4-blocking antibody rejected fully mismatched BALB/c cardiac grafts, whilst administration of CTLA4-blocking antibody in IL-6-deficient mice resulted in allograft acceptance. The authors suggested that blocking IL-6 production and IL-6 signalling with combined blockage of costimulatory molecules could inhibit Th1 responses, and promote transplant tolerance (Zhao et al. 2012). Therefore, given my findings that Treg EVs suppressed the production of IL-6 it could be a means to prevent transplant rejection and warrants further investigation.

Furthermore, there is a delicate balance between Th17 and Tregs. A signature transcription factor, RORγT for Th17 is also induced by TGF-β1. In the absence of IL-6, FoxP3 can inhibit RORγT function and drive Treg differentiation. However, when the cells also receive a signal from IL-6, FoxP3 function is inhibited and the Th17 differentiation pathway is induced (Z. Cai et al. 2012; Ziegler and Buckner 2009). Thus, the observation that Treg EVs but not Teff EVs significantly inhibited the production of IL-6 by Tresps highlights a possibility that Treg EVs are favouring FoxP3 function and thus possibly inducing T cell differentiation into induced Tregs, although this has not been tested directly here in this PhD project.

4.5 Summary

In summary, in this chapter, I demonstrated that both human Tregs and Teffs release EVs that inhibit Tresps proliferation. Treg EVs and Teff EVs act differently in regard to affecting cytokine release by Tresps. In particular, Treg EVs significantly inhibited IFNγ and IL-6 production levels by Tresps, whereas Teff EVs promoted their release. However, both Treg EVs and Teff EVs inhibited the production levels of IL-2, whilst increasing the production levels of IL-10, IL-4 and TNF by Tresps. In conclusion, human Tregs and Teffs release immunomodulatory EVs capable of inhibiting Tresps proliferation and altering their cytokine production profile.

Chapter 5

Investigation of miRNAs contained in Human Treg EVs

Chapter 5 - Investigation of miRNAs contained in Human Treg EVs

5.1 Introduction

miRNA are small single stranded RNA molecules approximately 22 nucleotides in length, which bind to target mRNA to direct post-transcriptional repression to inhibit their translation into protein (Y. Cai et al. 2009). 3' UTR is the section of mRNA which comprises the regulatory regions that post-transcriptionally affect gene expression and is the location where miRNA binds to repress or degrade them. Approximately half of all protein-coding genes are regulated by miRNA (Friedman et al. 2008) equating to more than 8000 genes (Asirvatham, Magner, and Tomasi 2009), thus miRNA plays important roles in regulating gene expression.

One miRNA can target one or more mRNAs and one mRNA can be targeted by one or more miRNAs (Y. Cai et al. 2009; Gebert and MacRae 2018). This level of interaction between miRNA and target mRNA can control the production of various proteins. Therefore, miRNAs are key bioactive molecules that can affect various immune cells; the absence or altered levels of miRNAs have resulted in immune system modification and dysfunction (Tsitsiou and Lindsay 2009; L.-F. Lu and Liston 2009). miRNAs not only have inhibitory effects on mRNA, which could be of therapeutic potential and they can be useful biomarkers to monitor health conditions or aid diagnosis and prognosis in patients (Jin Wang, Chen, and Sen 2016; K. W. Witwer 2015; Kreth, Hübner, and Hinske 2018; Hamam et al. 2017). It is therefore not surprising that miRNA is currently being considered for use in clinical trials (Chakraborty et al. 2017; Rupaimoole and Slack 2017).

5.1.1 miRNA and the immune system

In the immune system, miRNA have been linked to many aspects of T cell differentiation, Treg stability, and APC functions (Mehta and Baltimore 2016; Tsitsiou and Lindsay 2009; L.-F. Lu and Liston 2009; C. Xiao and Rajewsky 2009). Thus, the miRNA pool within the microenvironment can regulate the skewing of T cell lineages. For example, the host laboratory (Tung et al. 2018) and others (Jeker and Bluestone

2013) describes that miRNA expression in T cells including Tregs, can be downregulated after TCR activation. Therefore, given that EVs are produced after Treg activation and that miRNAs are shuttled into the Treg EVs, as shown in Chapter 3 with mouse Tregs, it would be reasonable to anticipate that human Tregs do the same as a mode of communication to other cell types.

Mittlebrunn *et al*. demonstrated that MVBs in jurkat T cells migrate toward the immunological synapse formed following recognition of antigen presented by APCs. These EVs harboured miRNAs, which were transferred in a unidirectional manner into APCs during an immunological synapsis; these miRNAs were functional and affected the target cell gene expression (Mittelbrunn et al. 2011).

It has been shown by various groups that miRNA expression can be induced by cytokines and cytokines can be influenced by miRNA. For example, IL-2 induces miR-182 expression and promotes IL-2-driven clonal Th expansion (Stittrich et al. 2010). In addition, IFNγ induces miR-29ab-1 expression in a regulatory feedback loop as miR-29ab-1 regulates T-bet and IFNγ directly, thus miR-29ab-1 regulates Th1 differentiation (Smith et al. 2012). Cytokines can be influenced by the presence of inhibitory miRNA as well. As demonstrated by Okoye *et al*., miRNA Let-7d contained in mouse Treg exosomes contributed to their suppression. Namely, this exosome-associated miRNA was able to function both *in vitro* to suppress Teffs proliferation and their cytokine production of IL-2 and IFNγ and *in vivo* to prevent inflammatory colitis in mouse models (Okoye et al. 2014).

5.1.2 miRNA and Tregs

EV-associated miRNAs not only affect Teffs but can also influence Tregs. For example, Kimura *et al.* investigated why patients with multiple sclerosis have a decreased frequency of Tregs. These authors found that circulating EVs from these patients contained high expression levels of Let-7i, and this miRNA targeted the IGF1R and TGFBR1 pathways which subsequently blocked the induction of Tregs, thus exacerbating the pathogenesis of multiple sclerosis disease (Kimura et al. 2018).

During my PhD project, the human Treg EVs miRNA contents had not been identified and had been one of my research aims. Recently a miRNOME of miRNA present in

human Treg EVs was published (Torri et al. 2017). In this report by Torri *et al*., the EVs derived from Th1, Th17 and Treg cells were assessed for their miRNA content. miR-146a-5p, miR-150-5p and miR-21-5p were identified as being enriched in Treg EVs. miR-146a-5p was noted by these authors to be the most probable miRNA contained in Treg EVs that drove immunosuppression of proliferating T cells. Two targets of miR-146a-5p; Stat1 and Irak2, were found at lower expression levels in T cells when the T cells were treated with Treg EVs. Given that Stat1 and Irak2 are two genes that are up-regulated upon stimulation of naïve T cells, Torri *et al*. suggested that the suppressed expression of these genes may have prevented their maturation (Torri et al. 2017). Taken together, miRNAs can modulate target cell biology. However, how the miRNA repertoire functions within human Treg EVs remain to be fully understood.

5.2 Aims and Objectives

Aim

As shown in Chapter 3, mouse Treg EVs contained miRNAs and these miRNAs may have contributed to their suppressive function. The original aim of this PhD thesis chapter was to investigate whether human Treg EVs also contained miRNAs and which of these miRNAs were likely to be involved in modulating the cytokine production by Tresps as shown in section 4.3.7.

Objectives:

(1) Define whether Treg EVs contain miRNAs
(2) Compare the miRNA repertoire of:
    - Tregs with Teffs
    - Tregs with Treg EVs
    - Teffs with Teff EVs
    - Treg EVs with Teff EVs
(3) Perform bioinformatic analysis to investigate whether miRNAs that are enriched in Treg EVs bind to and target 3' UTR of IFNγ, IL-2 and IL-6 mRNA
(4) Perform bioinformatic analysis to investigate which miRNAs are *in silico* predicted to target KEGG pathways related to immune signalling pathways.

5.3 Results

5.3.1 Human Tregs, Teffs, Treg EVs and Teff EVs contain different miRNA repertoires

In section 3.3.8, I have shown that mouse Treg EVs and FoxP3^{low} T cell EVs express different miRNA repertoires. As abovementioned, my original aim of this PhD thesis chapter was to investigate whether human Treg EVs contained miRNAs. Given that in Chapter 3, I demonstrated that murine Treg EVs affect the cytokine production by BM-DCs and given that EV-associated miRNA can mediate alteration in target cell production of cytokines (Okoye et al. 2014), it led to the question of whether the suppressive ability of human Treg EVs was possibly due to their miRNA content.

Thus, to investigate this, the miRNA repertoire in human Tregs, Treg EVs, autologous Teffs and Teff EVs were derived from 3 healthy donors and their total RNA isolated and the miRNA content assessed.

Due to this PhD project's main interest in Treg EVs over the other sample types (Tregs, Teffs and Teff EVs); Treg EVs were used as the main comparison for the subsequent analysis. Noteworthy, due to human to human variation, not all 3 donors would express miRNA-x but if at least one donor had a positive detection of miRNA-x in the RNA sample, it was recorded as present within that sample and this rule was applied to generate the following results.

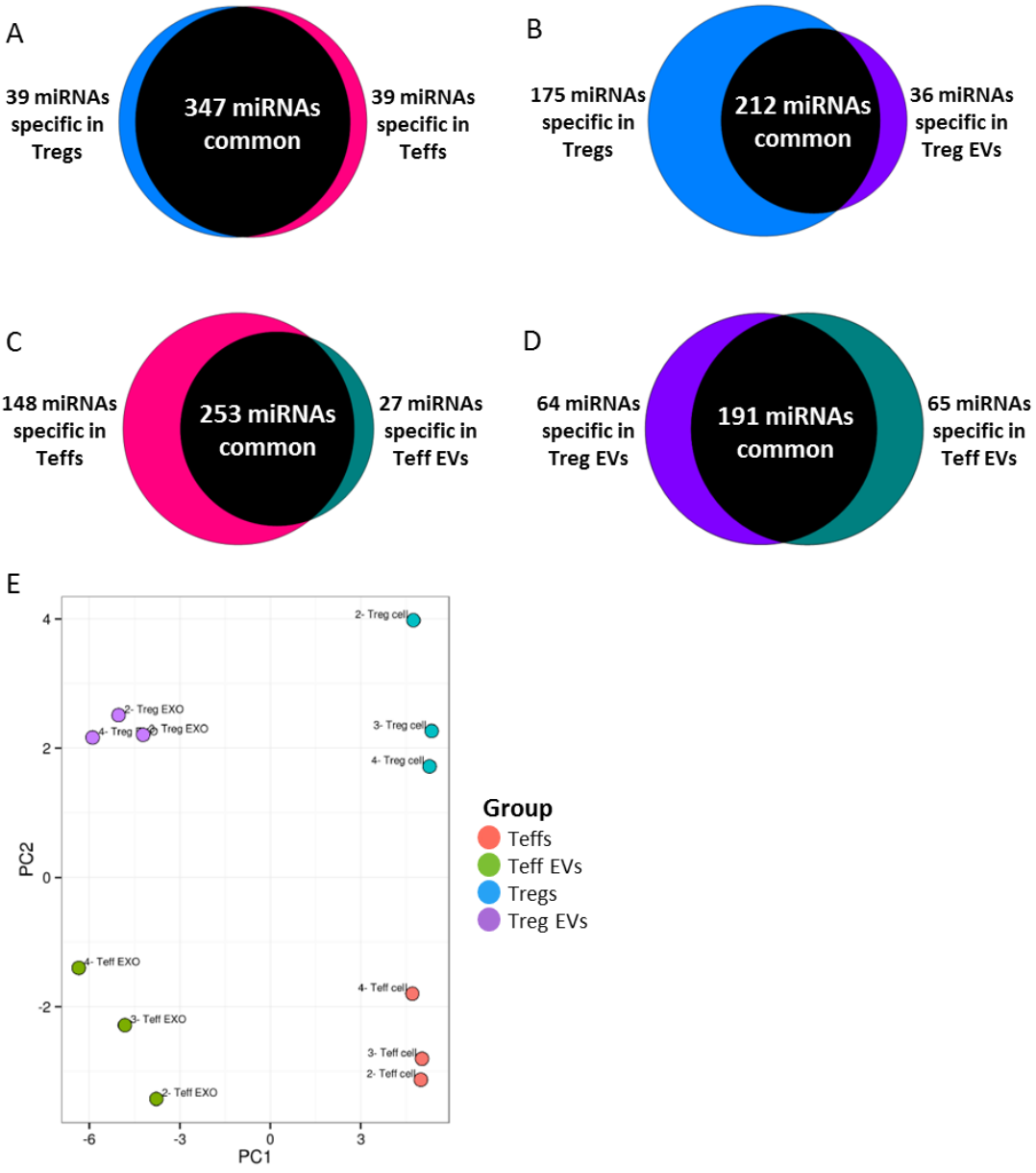
The miRNA content in each of the sample type was different; some miRNAs were found exclusively in cells or EVs. The Venn diagrams illustrated in Figure 5.1-A-D show the scaled proportion of the commonly and exclusively expressed miRNAs between two sample types. When comparing Tregs with Teffs, 347 miRNAs were commonly expressed between the two cell types, whereas 39 miRNAs were found exclusively in Tregs or Teffs (Figure 5.1-A).

212 miRNAs were found to be commonly expressed by Tregs and Treg EVs; whilst 175 miRNAs were found exclusively in parent Tregs and 36 miRNAs were found exclusively in Treg EVs (Figure 5.1-B). Teffs and Teff EVs had 253 miRNAs that were commonly expressed, moreover, 148 miRNAs were specific to Teffs and 27 miRNAs were specific to Teff EVs (Figure 5.1-C). The comparison of Treg EVs with Teff EVs highlighted that 191

miRNAs were common whilst there were 64 miRNAs found exclusively in Treg EVs and 65 miRNAs found exclusively in Teff EVs (Figure 5.1-D).

With the top 50 miRNAs that had the largest variation amongst the sample types, a principal component analysis (PCA) plot was used to assess their biological grouping based on their clustering profile. From the PCA plot, it was observed that in all 3 donor samples of Tregs, Teffs, Treg EVs and Teff EVs, the dot plots clustered together and in distinct regions of the PCA plot which indicates that their miRNA differences are likely due to their biological content rather than other technical factors such as poor-quality RNA input (Figure 5.1-E). For example, if Donor '2' Treg EVs had poor quality RNA used in the assays, then Donor '2' Treg EVs will not cluster according to their biology and would be separated notably further from Donor '3' or '4' Treg EVs.

Within the sample types of Tregs, Teffs, Treg EVs and Teff EVs there were commonly expressed miRNAs. The top 50 miRNAs that are commonly expressed in all the sample types (the sample set) and with the highest standard deviation are showed in the heat map in Figure 5.1-F. The heat map shows the two-way hierarchical clustering of miRNA and sample types, with the miRNA clustering tree on the left. When comparing the sample types together, cells and their EVs have diverse expression levels of particular miRNAs. Some miRNAs are found higher expressed in EVs than their parent cells and vice versa (Figure 5.1-F). However, to understand these differences better, a comparison of two samples rather than four sample types was required and described below.


A
39 miRNAs specific in Tregs
347 miRNAs common
39 miRNAs specific in Teffs
B
175 miRNAs specific in Tregs
212 miRNAs common
36 miRNAs specific in Treg EVs
C
148 miRNAs specific in Teffs
253 miRNAs common
27 miRNAs specific in Teff EVs
D
64 miRNAs specific in Treg EVs
191 miRNAs common
65 miRNAs specific in Teff EVs
E
2- Treg cell
2- Treg EXO
4- Treg EXO
3- Treg cell
4- Treg cell
4- Teff EXO
4- Teff cell
3- Teff EXO
3- Teff cell
2- Teff cell
2- Teff EXO
PC2
PC1
4
2
0
-2
-6
-3
0
3
Group
Teffs
Teff EVs
Tregs
Treg EVs

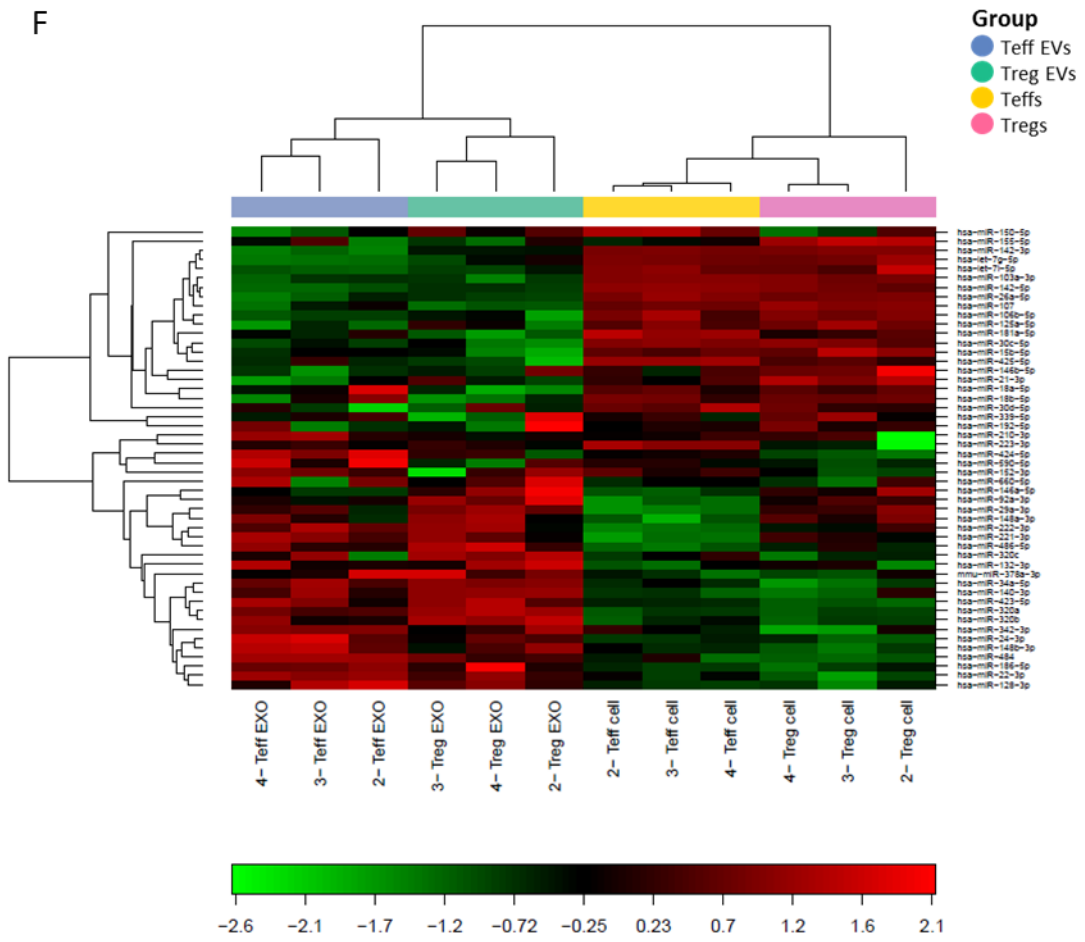


Figure 5.1- The miRNA repertoire in Tregs, Teffs, Treg EVs and Teff EVs

Venn diagrams drawn to scale to show the number of miRNAs that are commonly or exclusively expressed in ***(A)*** *Tregs and Teffs* ***(B)*** *Tregs and Treg EVs* ***(C)*** *Teffs and Teff EVs and* ***(D)*** *Treg EVs and Teff EVs.* ***(E)*** *Principal component analysis (PCA) plot showing the biological variation and clustering profile analysis performed on the top 50 miRNAs with the highest standard deviation in the sample set of Tregs (blue), Treg EVs (purple), Teffs (pink) and Teff EVs (green). The normalised (dCq) values have been used for the analysis.* ***(F)*** *Heatmap diagram and unsupervised hierarchical clustering of the top 50 miRNAs with the highest standard deviation in the sample set of Tregs, Teffs, Treg EVs and Teff EVs. The heatmap shows the result of the two-way hierarchical clustering of miRNAs and samples. Each row represents one miRNA whilst each column is one sample. The miRNA clustering tree is depicted on the left of the heatmap. In the colour scale, red represents a relative high expression of miRNA compared to green which is a lower expression level of miRNA than the mean in all the samples. Normalisation tool used was the global mean normalisation.*

5.3.2 Differential expression of miRNAs between Tregs and Teffs

Tregs and Teffs function differently, which may be due to their different miRNA content (Smigielska-Czepiel et al. 2014; Ha 2011). Since human samples used for experimental assays can vary considerably due to the genetic variation, it was important to identify the various miRNAs which were contained in the parent cells or their EVs. Thus, to first understand which miRNAs are present in Tregs and Teffs and to compare their expression levels, a heat-map diagram is displayed in Figure 5.2-A. The associated volcano plot is showed in Figure 5.2-B. To visualise this in a text format, Table 5.1 lists the miRNAs that are most differentially expressed between Tregs and Teffs, ranked according their t-test p-value. For example, miR-146a-5p, miR-21-3p, miR-21-5p and miR-155-5p are higher expressed in Tregs than Teffs. Although there was a trend of increase of miR-142-3p expression levels in Teffs compared to Tregs, this was not significant (Figure 5.2-C). Nonetheless, individual qPCR assays validated the miRNOME finding that Teffs expressed higher levels of miR-150-5p than in Tregs (Figure 5.2-D).

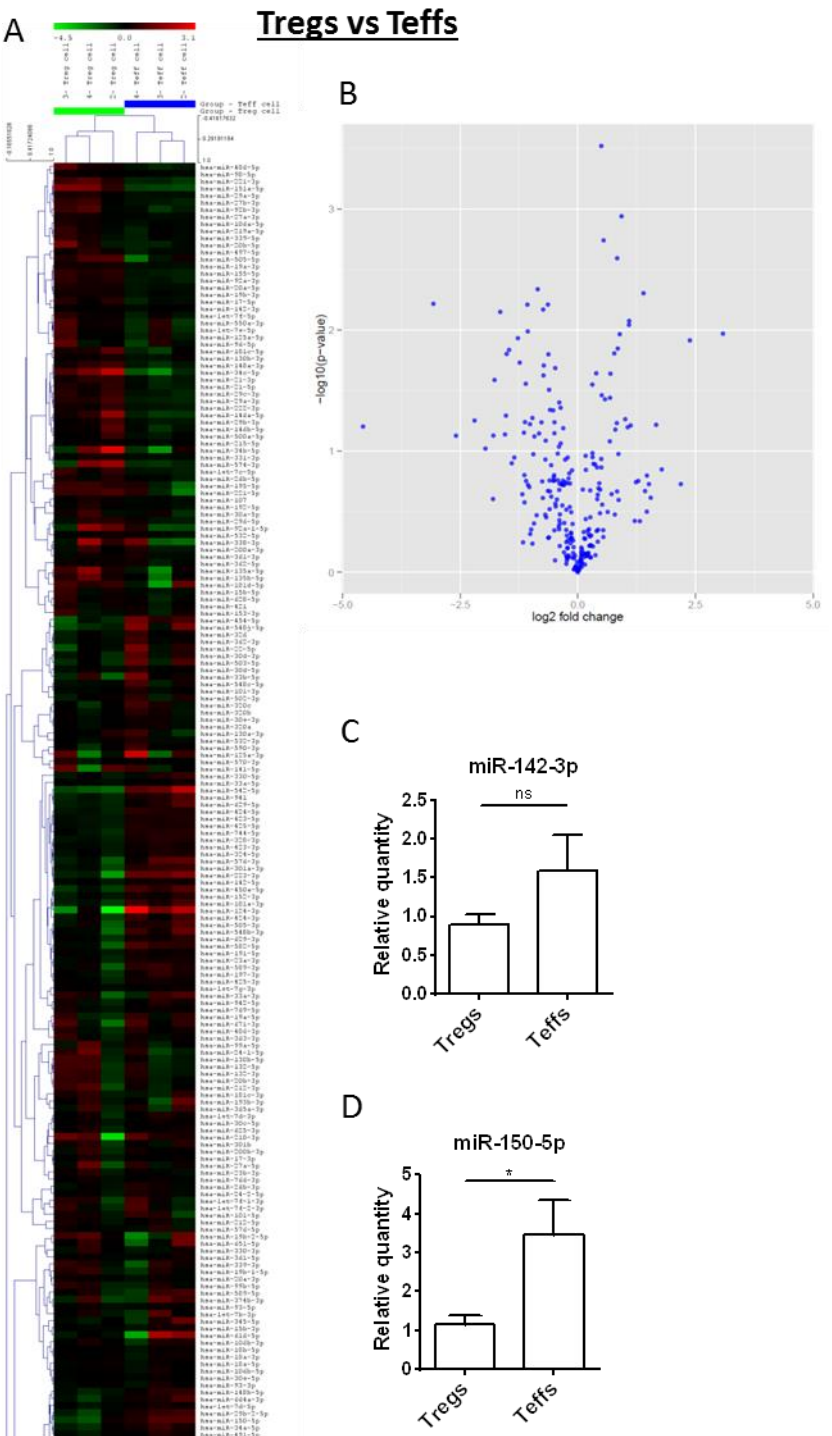

A
Tregs vs Teffs
B
-log10(p-value)
log2 fold change
C
miR-142-3p
ns
Relative quantity
Tregs
Teffs
D
miR-150-5p
*
Relative quantity
Tregs
Teffs

Figure 5.2- Tregs and Teffs contain different miRNA repertoires

(A) *The heatmap diagram shows the two-way unsupervised hierarchical clustering of miRNAs and Tregs (first three columns) and Teffs (last three columns).* ***(B)*** *Volcano plot displaying the numbers of miRNAs differentially expressed between Tregs and Teffs. Validation individual qPCR assays showing the expression levels of Tregs compared to Teffs for* ***(C)*** *miR-142-3p and* ***(D)*** *miR-150-5p. Student t-test was used to test significance. *p<0.05 and ns= non-significant*

miRNA	Fold change Tregs to Teffs	t-test p-value	Benjamini-Hochberg FDR
hsa-miR-19a-3p	1.413314518	0.000301854	0.077274515
hsa-miR-155-5p	1.905238923	0.001149001	0.147072105
hsa-miR-20a-5p	1.462564833	0.001817306	0.155076779
hsa-miR-92a-3p	1.785910049	0.002553739	0.163439286
hsa-miR-424-5p	-1.805132894	0.004601595	0.164762871
hsa-miR-221-3p	2.633130914	0.004962544	0.164762871
hsa-miR-542-5p	-8.390895915	0.006068729	0.164762871
hsa-miR-423-5p	-1.552357627	0.006155939	0.164762871
hsa-miR-7-5p	-2.101132926	0.006168189	0.164762871
hsa-miR-328-3p	-1.664885016	0.006766171	0.164762871
hsa-miR-181b-5p	-3.135749713	0.007079655	0.164762871
hsa-miR-148a-3p	2.131021135	0.008406853	0.17297185
hsa-miR-29a-3p	2.126556832	0.009060746	0.17297185
hsa-miR-941	-2.089111512	0.010256588	0.17297185
hsa-miR-34c-5p	8.482797531	0.010700425	0.17297185
hsa-miR-29c-3p	1.855371348	0.010810741	0.17297185
hsa-miR-181a-5p	-2.417170179	0.011664638	0.173114274
hsa-miR-151a-5p	5.20863425	0.012172097	0.173114274
hsa-miR-21-3p	1.803172249	0.014201982	0.176528635
hsa-miR-1260a	-2.751355064	0.014585201	0.176528635
hsa-miR-21-5p	1.711399188	0.015571575	0.176528635
hsa-miR-450a-5p	-2.851651819	0.015735626	0.176528635
hsa-miR-425-5p	-1.541045019	0.015859995	0.176528635
hsa-miR-181a-3p	-2.352371568	0.018564098	0.198017041
hsa-miR-148b-5p	-1.649967715	0.01960649	0.200770456
hsa-miR-423-3p	-1.394682293	0.02060202	0.202850655
hsa-miR-19b-3p	1.311636024	0.022744983	0.209084659
hsa-miR-17-5p	1.618043183	0.022907207	0.209084659
hsa-miR-185-5p	-1.657828564	0.023685371	0.209084659
hsa-miR-454-5p	-3.408183968	0.025824942	0.220372839
hsa-miR-940	-2.15230609	0.027740112	0.225514625
hsa-miR-107	1.238502263	0.028189328	0.225514625
hsa-miR-152-3p	-1.525654637	0.031154225	0.241681259
hsa-miR-29a-5p	1.422665256	0.034529352	0.259985712
hsa-miR-222-3p	1.60854774	0.036249999	0.264905931
hsa-miR-497-5p	1.493174205	0.037252396	0.264905931
hsa-miR-590-5p	-1.313776388	0.039608748	0.274049715
hsa-miR-34a-3p	-1.287687634	0.043795863	0.294274834
hsa-miR-29c-5p	-1.50541515	0.045572658	0.294274834
hsa-miR-324-5p	-1.435935751	0.045980443	0.294274834
hsa-miR-576-3p	-2.881794163	0.050844646	0.305987775
hsa-miR-664a-3p	-1.950920662	0.053210566	0.305987775
hsa-miR-486-5p	2.011640775	0.054410352	0.305987775
hsa-miR-1972	-4.575921741	0.055801129	0.305987775
hsa-miR-29b-2-5p	-2.175839558	0.057503488	0.305987775
hsa-miR-148b-3p	-1.409568053	0.057717384	0.305987775
hsa-miR-744-5p	-1.707481537	0.057790176	0.305987775
hsa-miR-27b-3p	1.776789392	0.058593065	0.305987775
hsa-miR-629-5p	-2.022625837	0.059755157	0.305987775
hsa-miR-146a-5p	3.170264851	0.060518189	0.305987775

Table 5.1- Table of top 50 miRNAs that are most differentially expressed by Tregs and Teffs.

The list is ranked according to the t-test p-value. FDR means false discovery rate.

miRNA name	Treg EVs Ct value	Tregs Ct value	miRNA reported in ExoCarta	Species	Tissue/Sample type	PubMed ID
miR-602	>30<40	ND	No	N/A	N/A	N/A
miR-376a-3p	>30<40	ND	Yes	Homo Sapiens	Plasma	23663360
miR-187-5p	>30<40	ND	Yes	Homo Sapiens	Plasma	23663360
miR-133b	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells	N/A
miR-369-3p	>30<40	ND	Yes	Homo Sapiens	Melanoma cells Plasma Serum	26176991 23663360 18589210
miR-143-3p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Melanoma cells Plasma	25330373 26176991 23663360
miR-122-5p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Plasma	N/A 23663360
miR-760	>30<40	ND	No	N/A	N/A	N/A
miR-144-5p	>30<40	ND	Yes	Homo Sapiens	Melanoma cells Plasma	26176991 23663360
miR-608	>30<40	ND	No	N/A	N/A	N/A
miR-596	>30<40	ND	No	N/A	N/A	N/A
miR-382-5p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Plasma	N/A 23663360
miR-376c-3p	>30<40	ND	Yes	Homo Sapiens	Melanoma cells Plasma	26176991 23663360
miR-346	>30<40	ND	No	N/A	N/A	N/A
miR-934	>30<40	ND	No	N/A	N/A	N/A
miR-885-5p	>30<40	ND	Yes	Homo Sapiens	B cells Colorectal cancer cells Colorectal cancer cells Plasma	21505438 N/A N/A 23663360
miR-885-3p	>30<40	ND	Yes	Homo Sapiens	Plasma	23663360
miR-765	>30<40	ND	No	N/A	N/A	N/A
miR-675-3p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Colorectal cancer cells	N/A N/A 25330373
miR-638	>30<40	ND	No	N/A	N/A	N/A
miR-609	>30<40	ND	No	N/A	N/A	N/A
miR-604	>30<40	ND	No	N/A	N/A	N/A
miR-584-5p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Colorectal cancer cells Plasma	N/A N/A 25330373 23663360
miR-517-5p	>30<40	ND	No	N/A	N/A	N/A
miR-502-5p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Dendritic Cells T cells	N/A 21505438 21505438
miR-376b-3p	>30<40	ND	Yes	Homo Sapiens	Plasma	23663360
miR-34c-3p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Plasma	N/A N/A 23663360
miR-329-3p	>30<40	ND	No	N/A	N/A	N/A
miR-218-1-3p	>30<40	ND	No	N/A	N/A	N/A
miR-206	>30<40	ND	No	N/A	N/A	N/A
miR-205-5p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Plasma	N/A N/A 23663360
miR-200b-5p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Plasma	N/A 25330373 23663360
miR-195-3p	>30<40	ND	Yes	Homo Sapiens	Plasma	23663360
miR-133a-3p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells	N/A
miR-1269a	>30<40	ND	No	N/A	N/A	N/A
miR-10a-3p	>30<40	ND	Yes	Homo Sapiens	Colorectal cancer cells Plasma	25330373 23663360

Table 5.2- Table of miRNAs found exclusively in Treg EVs and not in Tregs.

The associated information as to whether these miRNAs are reported in ExoCarta is also included.

5.3.3 Human Treg EVs contain exclusive miRNAs that are not found in their parent cells

To investigate which specific miRNAs contained in Treg EVs may be contributing to their suppressive function that is not otherwise found in their parent Tregs, the miRNA content of Treg EVs was compared to their parent Tregs.

As displayed in **Figure 5.1-B**, 36 miRNAs were found exclusively in Treg EVs but not Tregs, which are detailed in **Table 5.2**, along with the associated information of whether these miRNAs have previously been reported in ExoCarta (a database of exosomes-related miRNAs) and the biological sample type from which that miRNA had been detected. For example, mir-369-3p, miR-376c-3p, miR-195-3p, miR-885-5p and miR-125b-5p are all found only in Treg EVs but not their parent Treg cells.

The heatmap diagram shows the two-way unsupervised hierarchical clustering of miRNAs of Treg EVs and their parent Tregs (**Figure 5.3-A**). The volcano plot displays the annotated top 20 miRNAs with the most significantly differentially expressed miRNAs between Treg EV and their parent Tregs (**Figure 5.3-B**), with the miRNAs appearing on the left side of the volcano plot being higher expressed in Treg EVs compared to Tregs and vice versa.

In **Table 5.3**, the top 22 miRNAs (ranked according to the p-value) that were higher expressed in Treg EVs compared to their parent Tregs (e.g. miR-1972, miR-126-3p and miR-451 were all >300 folds higher expressed in Treg EVs than Tregs) are listed and in **Table 5.4** the miRNAs that were the most differentially expressed between Treg EVs and their parent Tregs and ranked according their t-test p-value are listed.

The miRNOME screen showed that Tregs expressed higher levels of miR-142-3p than in Treg EVs, whereas the expression levels of miR-150-5p may be similar, to validate this observation, individual qPCR assays were performed. As shown by individual qPCR assays, Tregs expressed higher levels of miR-142-3p than Treg EVs (**Figure 5.3-C**), although no statistical difference was observed for miR-150-5p expression levels (**Figure 5.3-D**).

Overall, this data demonstrated that Treg EVs contain exclusive miRNAs and also contain some miRNAs which are expressed at higher levels compared to their parent Tregs.

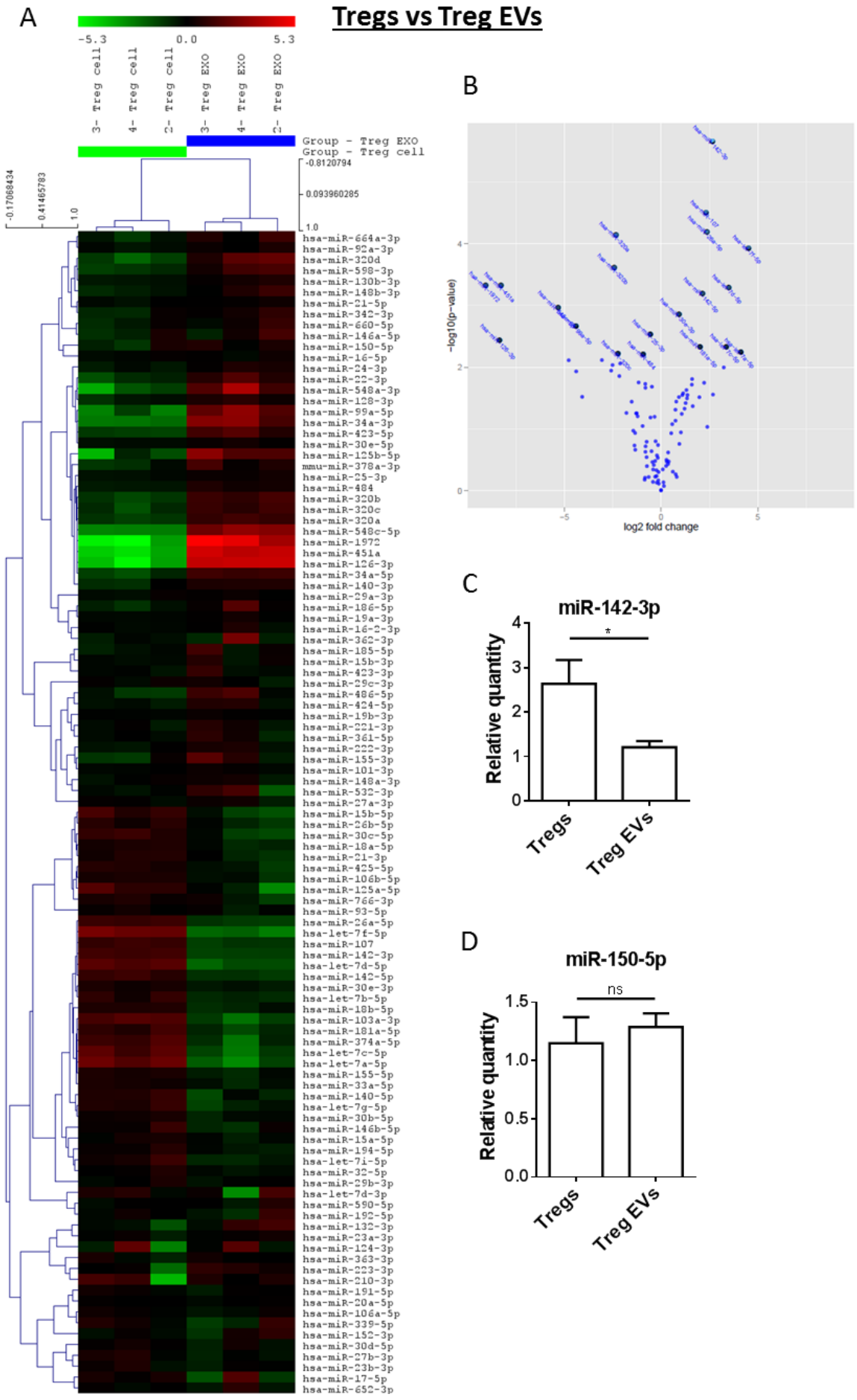

A
Tregs vs Treg EVs
-5.3
0.0
5.3
3- Treg cell
4- Treg cell
2- Treg cell
3- Treg EXO
4- Treg EXO
2- Treg EXO
Group - Treg EXO
Group - Treg cell
-0.8120794
0.093960285
1.0
-0.17068434
0.41465783
1.0
hsa-miR-664a-3p
hsa-miR-92a-3p
hsa-miR-320d
hsa-miR-598-3p
hsa-miR-130b-3p
hsa-miR-148b-3p
hsa-miR-21-5p
hsa-miR-342-3p
hsa-miR-660-5p
hsa-miR-146a-5p
hsa-miR-150-5p
hsa-miR-16-5p
hsa-miR-24-3p
hsa-miR-22-3p
hsa-miR-548a-3p
hsa-miR-128-3p
hsa-miR-99a-5p
hsa-miR-34a-3p
hsa-miR-423-5p
hsa-miR-30e-5p
hsa-miR-125b-5p
mmu-miR-378a-3p
hsa-miR-25-3p
hsa-miR-484
hsa-miR-320b
hsa-miR-320c
hsa-miR-320a
hsa-miR-548c-5p
hsa-miR-1972
hsa-miR-451a
hsa-miR-126-3p
hsa-miR-34a-5p
hsa-miR-140-3p
hsa-miR-29a-3p
hsa-miR-186-5p
hsa-miR-19a-3p
hsa-miR-16-2-3p
hsa-miR-362-3p
hsa-miR-185-5p
hsa-miR-15b-3p
hsa-miR-423-3p
hsa-miR-29c-3p
hsa-miR-486-5p
hsa-miR-424-5p
hsa-miR-19b-3p
hsa-miR-221-3p
hsa-miR-361-5p
hsa-miR-222-3p
hsa-miR-155-3p
hsa-miR-101-3p
hsa-miR-148a-3p
hsa-miR-532-3p
hsa-miR-27a-3p
hsa-miR-15b-5p
hsa-miR-26b-5p
hsa-miR-30c-5p
hsa-miR-18a-5p
hsa-miR-21-3p
hsa-miR-425-5p
hsa-miR-106b-5p
hsa-miR-125a-5p
hsa-miR-766-3p
hsa-miR-93-5p
hsa-miR-26a-5p
hsa-let-7f-5p
hsa-miR-107
hsa-miR-142-3p
hsa-let-7d-5p
hsa-miR-142-5p
hsa-miR-30e-3p
hsa-let-7b-5p
hsa-miR-18b-5p
hsa-miR-103a-3p
hsa-miR-181a-5p
hsa-miR-374a-5p
hsa-let-7c-5p
hsa-let-7a-5p
hsa-miR-155-5p
hsa-miR-33a-5p
hsa-miR-140-5p
hsa-let-7g-5p
hsa-miR-30b-5p
hsa-miR-146b-5p
hsa-miR-15a-5p
hsa-miR-194-5p
hsa-let-7i-5p
hsa-miR-32-5p
hsa-miR-29b-3p
hsa-let-7d-3p
hsa-miR-590-5p
hsa-miR-192-5p
hsa-miR-132-3p
hsa-miR-23a-3p
hsa-miR-124-3p
hsa-miR-363-3p
hsa-miR-223-3p
hsa-miR-210-3p
hsa-miR-191-5p
hsa-miR-20a-5p
hsa-miR-106a-5p
hsa-miR-339-5p
hsa-miR-152-3p
hsa-miR-30d-5p
hsa-miR-27b-3p
hsa-miR-23b-3p
hsa-miR-17-5p
hsa-miR-652-3p
B
-log10(p-value)
log2 fold change
C
miR-142-3p
Relative quantity
*
Tregs
Treg EVs
D
miR-150-5p
Relative quantity
ns
Tregs
Treg EVs

Figure 5.3- Tregs and Treg EVs contain different miRNA repertoires

(A) *The heatmap diagram shows the two-way unsupervised hierarchical clustering of miRNAs and Treg EVs (last three columns) and their parent Tregs (first three columns), where each row represents one miRNA and each column represents the indicated sample type. The miRNA clustering tree is also indicated with numerical values. Green indicates low expression levels whereas red indicates high expression levels.* ***(B)*** *Volcano plot displaying the annotated top 20 miRNAs with the most significant differentially expressed miRNAs between Treg EVs and their parent Tregs. The miRNAs appearing on the left side of the volcano plot are miRNAs higher expressed in Treg EVs compared to right side of the volcano plot, Tregs. The miRNA appearing at the right side of the volcano plot are higher expressed in parent Tregs compared to Treg EVs. Validation individual qPCR assays showing the expression levels of Tregs compared to Treg EVs for* ***(C)*** *miR-142-3p and* ***(D)*** *miR-150-5p. Student t-test was used to test significance. *p<0.05 and ns= non-significant.*

miRNA name	Fold change Treg EVs/Tregs	t-test p-value	Benjamini-Hochberg FDR	miRNA reported in ExoCarta	Species	Tissue/Sample type	PubMed ID
miR-320b	5.36	0.000240837	0.004294927	Yes	Homo Sapiens - Equus caballus	Colorectal cancer cells Plasma Follicular Fluid	25330373 23663360 22116803
miR-1972	536.20	0.000469308	0.006017836	No	N/A	N/A	N/A
miR-451a	311.50	0.000471634	0.006017836	Yes	Homo Sapiens	Colorectal cancer cells Melanoma cells Plasma	25330373 26176991 23663360
miR-548c-5p	40.25	0.001086231	0.010566065	No	N/A	N/A	N/A
miR-99a-5p	21.24	0.002121807	0.0174641	Yes	Homo Sapiens	Plasma	23663360
miR-25-3p	1.47	0.002915005	0.02227897	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Colorectal cancer cells Plasma	N/A N/A 25330373 23663360
miR-126-3p	327.89	0.003646443	0.026011293	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Colorectal cancer cells Plasma	N/A N/A 25330373 23663360
miR-320c	4.70	0.006006923	0.032438531	Yes	Homo Sapiens - Equus caballus	Colorectal cancer cells Plasma Follicular Fluid	25330373 23663360 22116803
miR-484	1.91	0.006063277	0.032438531	No	N/A	N/A	N/A
miR-320d	8.36	0.007672477	0.037526202	Yes	Homo Sapiens	Colorectal cancer cells Plasma	25330373 23663360
miR-34a-3p	27.47	0.007715668	0.037526202	Yes	Homo Sapiens	Plasma	23663360
miR-22-3p	4.34	0.008699645	0.04047226	Yes	Homo Sapiens	Colorectal cancer cells Plasma	25330373 23663360
miR-34a-5p	5.71	0.009225087	0.041128514	Yes	Homo Sapiens	Colorectal cancer cells Plasma	25330373 23663360
miR-423-5p	6.00	0.011356665	0.046400252	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Colorectal cancer cells	N/A N/A 25330373
miR-548a-3p	21.03	0.011708475	0.046400252	No	N/A	N/A	N/A
miR-598-3p	4.48	0.01392848	0.053226693	No	N/A	N/A	N/A
miR-128-3p	2.24	0.017673941	0.061800563	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells	N/A 25330373
miR-24-3p	1.77	0.018645449	0.062230157	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Colorectal cancer cells Plasma	N/A N/A 25330373 23663360
miR-30e-5p	1.40	0.020718435	0.065202134	Yes	Homo Sapiens	Colorectal cancer cells Plasma	25330373 23663360
miR-130b-3p	1.76	0.021562289	0.065918999	Yes	Homo Sapiens	Colorectal cancer cells Colorectal cancer cells Colorectal cancer cells Plasma	N/A N/A 25330373 23663360
miR-125b-5p	16.97	0.030237375	0.076271055	Yes	Homo Sapiens	Plasma	23663360
miR-378a-3p	2.88	0.036250096	0.084320876	Yes	Homo Sapiens	Colorectal cancer cells Plasma	25330373 23663360

Table 5.3- Table of the top 22 miRNAs that were higher expressed in Treg EVs compared to their parent Tregs.

The list is ranked according to the t-test p-value. FDR means false discovery rate.

miRNA	Fold change Treg EVs to Tregs	t-test p-value	Benjamini-Hochberg FDR
hsa-miR-142-3p	-6.24555013402685	2.15E-06	0.000230023
hsa-miR-107	-4.97547678934907	3.07E-05	0.001644362
hsa-miR-26a-5p	-5.1510374153722	6.35E-05	0.001914099
hsa-miR-320a	5.043312413	7.16E-05	0.001914099
hsa-let-7f-5p	-22.923254886939	0.000115825	0.002478651
hsa-miR-320b	5.363786824	0.000240837	0.004294927
hsa-miR-1972	536.2041719	0.000469308	0.006017836
hsa-miR-451a	311.4972689	0.000471634	0.006017836
hsa-let-7d-5p	-11.2194570813883	0.000506173	0.006017836
hsa-miR-142-5p	-4.40773694494717	0.000633278	0.006776074
hsa-miR-548c-5p	40.25065003	0.001086231	0.010566065
hsa-miR-30e-3p	-1.89824631739953	0.001387203	0.012369229
hsa-miR-99a-5p	21.23968459	0.002121807	0.0174641
hsa-miR-25-3p	1.472822713	0.002915005	0.02227897
hsa-miR-126-3p	327.8891306	0.003646443	0.026011293
hsa-let-7c-5p	-10.3309359791692	0.004654476	0.029518582
hsa-miR-181a-5p	-4.04018210771861	0.004689868	0.029518582
hsa-let-7a-5p	-17.3949853161932	0.005588373	0.032438531
hsa-miR-320c	4.702991269	0.006006923	0.032438531
hsa-miR-484	1.91167977	0.006063277	0.032438531
hsa-miR-320d	8.364473158	0.007672477	0.037526202
hsa-miR-34a-3p	27.47355118	0.007715668	0.037526202
hsa-miR-22-3p	4.343208976	0.008699645	0.04047226
hsa-miR-34a-5p	5.712868943	0.009225087	0.041128514
hsa-miR-103a-3p	-9.43036446528997	0.010096539	0.043213186
hsa-miR-423-5p	5.998635445	0.011356665	0.046400252
hsa-miR-548a-3p	21.03119299	0.011708475	0.046400252
hsa-miR-598-3p	4.478958495	0.01392848	0.053226693
hsa-let-7b-5p	-3.03111398571247	0.015571422	0.057453178
hsa-miR-128-3p	2.244604168	0.017673941	0.061800563
hsa-miR-15b-5p	-4.65842979945997	0.017904836	0.061800563
hsa-miR-24-3p	1.766166674	0.018645449	0.062230157
hsa-let-7g-5p	-2.99916007580199	0.019192478	0.062230157
hsa-miR-30e-5p	1.39552414	0.020718435	0.065202134
hsa-miR-130b-3p	1.757368168	0.021562289	0.065918999
hsa-miR-18b-5p	-2.47395136005875	0.023486155	0.069806073
hsa-miR-18a-5p	-2.1938387140088	0.026525218	0.07583157
hsa-miR-374a-5p	-6.40883382522899	0.027762419	0.07583157
hsa-let-7i-5p	-2.565579360302	0.028231502	0.07583157
hsa-miR-155-5p	-2.13498130365522	0.02834825	0.07583157
hsa-miR-125b-5p	16.97062439	0.030237375	0.076271055
hsa-miR-33a-5p	-1.88979675900161	0.030517775	0.076271055
hsa-miR-30c-5p	-3.92569104494944	0.030650985	0.076271055
hsa-miR-26b-5p	-2.52730102463857	0.031988111	0.07778927
hsa-miR-194-5p	-1.71778042500384	0.036103184	0.084320876
mmu-miR-378a-3p	2.880611732	0.036250096	0.084320876
hsa-miR-425-5p	-2.40180767105328	0.038162708	0.08688106
hsa-miR-140-5p	-3.10013547597412	0.042842556	0.095503199
hsa-miR-21-3p	-2.31100253020276	0.047063213	0.102770689
hsa-miR-30b-5p	-1.66694351166469	0.048866655	0.104574643

Table 5.4- Table of the top 50 miRNAs that are differentially expressed by Treg EVs and their parent Tregs.

The list is ranked according to the t-test p-value. FDR means false discovery rate.

5.3.4 Differential expression of miRNA between Teffs and Teff EVs

The same analysis performed with Tregs and Treg EVs was extended to Teffs and their EVs, as it has previously been reported that Teffs and their EVs differ in their miRNA content (Torri et al. 2017). To further confirm these findings, a comparison of the relative miRNA expression levels between Teffs and Teffs EVs was performed. A heatmap of the different miRNA expression levels is illustrated in **Figure 5.4-A**, with the associated volcano plot (**Figure 5.4-B**). **Table 5.5** lists the top 50 miRNAs that are most differentially expressed between Teffs and Teff EVs, ranked according their t-test p-value. Individual qPCR assays validated the miRNOME finding that Teffs expressed higher levels of miR-142-3p and miR-150-5p than in Teff EVs (**Figures 5.4-C and D**, respectively).

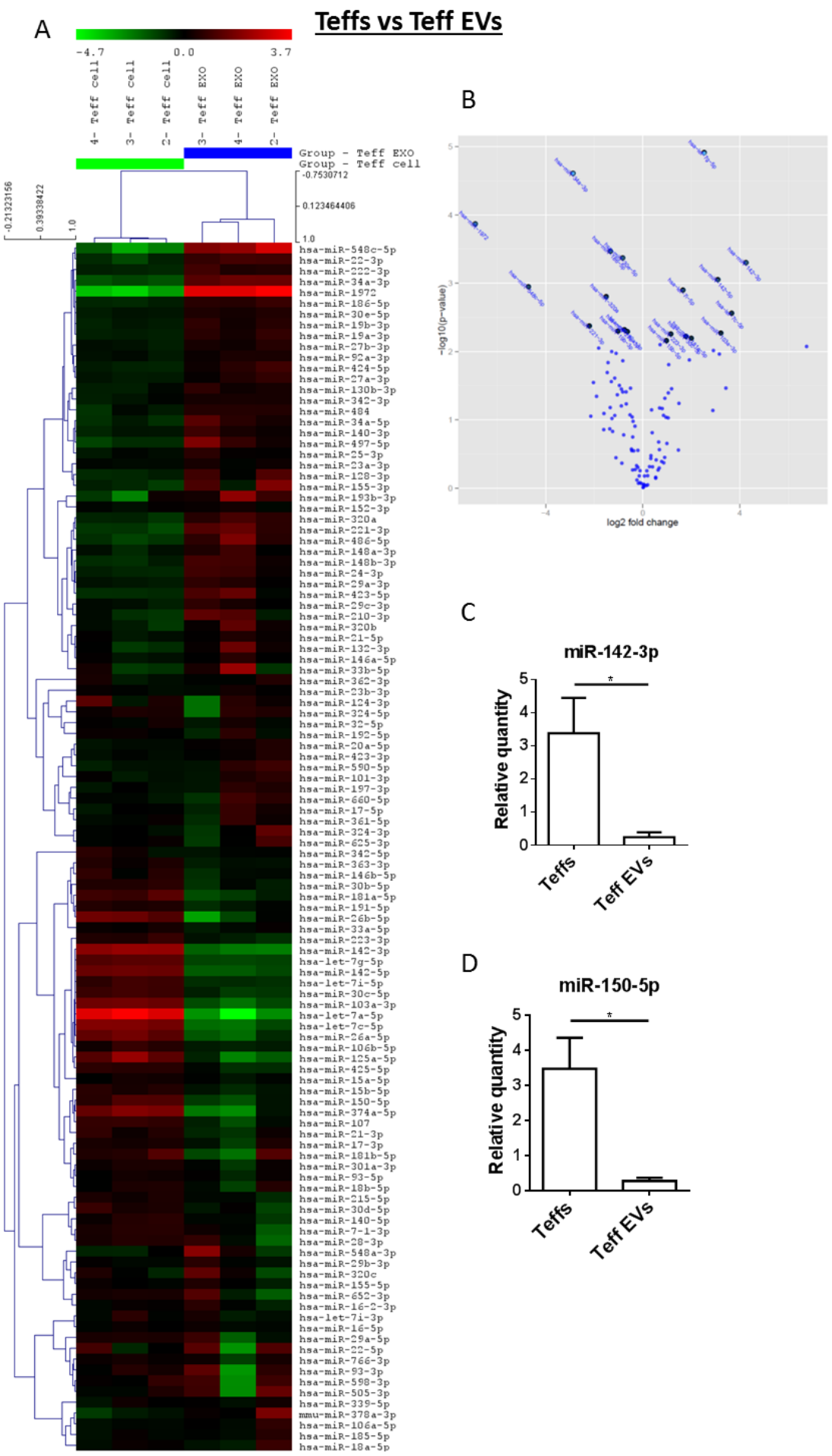

A
Teffs vs Teff EVs
-4.7
0.0
3.7
4- Teff cell
3- Teff cell
2- Teff cell
3- Teff EXO
4- Teff EXO
2- Teff EXO
Group - Teff EXO
Group - Teff cell
-0.7530712
0.123464406
1.0
-0.21323156
0.39338422
1.0
hsa-miR-548c-5p
hsa-miR-22-3p
hsa-miR-222-3p
hsa-miR-34a-3p
hsa-miR-1972
hsa-miR-186-5p
hsa-miR-30e-5p
hsa-miR-19b-3p
hsa-miR-19a-3p
hsa-miR-27b-3p
hsa-miR-92a-3p
hsa-miR-424-5p
hsa-miR-27a-3p
hsa-miR-130b-3p
hsa-miR-342-3p
hsa-miR-484
hsa-miR-34a-5p
hsa-miR-140-3p
hsa-miR-497-5p
hsa-miR-25-3p
hsa-miR-23a-3p
hsa-miR-128-3p
hsa-miR-155-3p
hsa-miR-193b-3p
hsa-miR-152-3p
hsa-miR-320a
hsa-miR-221-3p
hsa-miR-486-5p
hsa-miR-148a-3p
hsa-miR-148b-3p
hsa-miR-24-3p
hsa-miR-29a-3p
hsa-miR-423-5p
hsa-miR-29c-3p
hsa-miR-210-3p
hsa-miR-320b
hsa-miR-21-5p
hsa-miR-132-3p
hsa-miR-146a-5p
hsa-miR-33b-5p
hsa-miR-362-3p
hsa-miR-23b-3p
hsa-miR-124-3p
hsa-miR-324-5p
hsa-miR-32-5p
hsa-miR-192-5p
hsa-miR-20a-5p
hsa-miR-423-3p
hsa-miR-590-5p
hsa-miR-101-3p
hsa-miR-197-3p
hsa-miR-660-5p
hsa-miR-17-5p
hsa-miR-361-5p
hsa-miR-324-3p
hsa-miR-625-3p
hsa-miR-342-5p
hsa-miR-363-3p
hsa-miR-146b-5p
hsa-miR-30b-5p
hsa-miR-181a-5p
hsa-miR-191-5p
hsa-miR-26b-5p
hsa-miR-33a-5p
hsa-miR-223-3p
hsa-miR-142-3p
hsa-let-7g-5p
hsa-miR-142-5p
hsa-let-7i-5p
hsa-miR-30c-5p
hsa-miR-103a-3p
hsa-let-7a-5p
hsa-let-7c-5p
hsa-miR-26a-5p
hsa-miR-106b-5p
hsa-miR-125a-5p
hsa-miR-425-5p
hsa-miR-15a-5p
hsa-miR-15b-5p
hsa-miR-150-5p
hsa-miR-374a-5p
hsa-miR-107
hsa-miR-21-3p
hsa-miR-17-3p
hsa-miR-181b-5p
hsa-miR-301a-3p
hsa-miR-93-5p
hsa-miR-18b-5p
hsa-miR-215-5p
hsa-miR-30d-5p
hsa-miR-140-5p
hsa-miR-7-1-3p
hsa-miR-28-3p
hsa-miR-548a-3p
hsa-miR-29b-3p
hsa-miR-320c
hsa-miR-155-5p
hsa-miR-652-3p
hsa-miR-16-2-3p
hsa-let-7i-3p
hsa-miR-16-5p
hsa-miR-29a-5p
hsa-miR-22-5p
hsa-miR-766-3p
hsa-miR-93-3p
hsa-miR-598-3p
hsa-miR-505-3p
hsa-miR-339-5p
mmu-miR-378a-3p
hsa-miR-106a-5p
hsa-miR-185-5p
hsa-miR-18a-5p
B
−log10(p-value)
log2 fold change
C
miR-142-3p
Relative quantity
*
Teffs
Teff EVs
D
miR-150-5p
Relative quantity
*
Teffs
Teff EVs

Figure 5.4- Teffs and Teff EVs contain different miRNA repertoires

(A) *The heatmap diagram shows the two-way unsupervised hierarchical clustering of miRNAs and Teffs (first three columns) and Teff EVs (last three columns).* ***(B)*** *Volcano plot displaying the annotated top 20 miRNAs with the most significant differentially expressed miRNAs between Teffs and Teff EVs. The miRNAs appearing on the left side of the volcano plot are miRNAs higher expressed in Teff EVs compared to right side of the volcano plot Teffs. The miRNA appearing at the right side of the volcano plot are higher expressed in parent Teffs compared to Teff EVs. Validation individual qPCR assays showing the expression levels of Teffs compared to Teff EVs for* ***(C)*** *miR-142-3p and* ***(D)*** *miR-150-5p. Student t-test was used to test significance. *p<0.05*

miRNA	Fold change Teffs to Teff EVs	t-test p-value	Benjamini-Hochberg FDR
hsa-let-7g-5p	5.764031076	1.23E-05	0.001373622
hsa-miR-34a-3p	-7.34352082	2.45E-05	0.001373622
hsa-miR-1972	-120.0202952	0.000135844	0.005071492
hsa-miR-186-5p	-2.519385516	0.000342693	0.009359513
hsa-miR-30e-5p	-1.777590194	0.000427832	0.009359513
hsa-miR-142-3p	19.04546843	0.000501402	0.009359513
hsa-miR-142-5p	8.51758465	0.000892726	0.014283608
hsa-miR-548c-5p	-26.2396222	0.001132572	0.015713425
hsa-let-7i-5p	3.138371234	0.001262686	0.015713425
hsa-miR-320a	-2.841826892	0.001584167	0.017742675
hsa-let-7c-5p	12.62541968	0.002763304	0.028135462
hsa-miR-221-3p	-4.592014518	0.004256473	0.036558722
hsa-miR-27b-3p	-1.690249761	0.004837301	0.036558722
hsa-miR-19b-3p	-2.030213385	0.005047221	0.036558722
hsa-miR-92a-3p	-1.551939879	0.005098216	0.036558722
hsa-miR-103a-3p	9.274290113	0.005401903	0.036558722
hsa-miR-223-3p	2.223171246	0.005549092	0.036558722
hsa-miR-30c-5p	3.430465047	0.0059801	0.037209512
hsa-miR-181a-5p	3.993922422	0.00638573	0.037642196
hsa-miR-15b-5p	1.959495152	0.00694302	0.038880913
hsa-miR-363-3p	1.640646079	0.007933275	0.042310798
hsa-let-7a-5p	107.7227419	0.008442815	0.042981606
hsa-miR-22-3p	-3.521391585	0.008909441	0.043385105
hsa-miR-26a-5p	7.519493218	0.009668981	0.044932536
hsa-miR-222-3p	-2.429999915	0.010029584	0.044932536
hsa-miR-19a-3p	-2.143328202	0.010615359	0.045173163
hsa-miR-125a-5p	8.91059566	0.010889959	0.045173163
hsa-miR-150-5p	3.788734252	0.01329641	0.052730784
hsa-miR-130b-3p	-1.767752096	0.013653507	0.052730784
hsa-miR-148b-3p	-2.696903219	0.014525074	0.054226942
hsa-miR-106b-5p	2.097612106	0.015579112	0.056285825
hsa-miR-34a-5p	-2.758634718	0.020321518	0.071125315
hsa-miR-424-5p	-2.226504699	0.024524114	0.083233356
hsa-miR-24-3p	-2.453421622	0.027556118	0.090773093
hsa-miR-486-5p	-4.092953755	0.028451752	0.091045605
hsa-miR-140-3p	-1.950901866	0.031378371	0.0976216
hsa-miR-374a-5p	10.74398326	0.034299583	0.099337044
hsa-miR-20a-5p	-1.418540227	0.034452477	0.099337044
hsa-miR-425-5p	2.276642216	0.035103458	0.099337044
hsa-miR-27a-3p	-1.84189176	0.035477516	0.099337044
hsa-miR-342-3p	-1.610196945	0.039027444	0.104164345
hsa-miR-29a-3p	-1.772772557	0.039061629	0.104164345
hsa-miR-30b-5p	2.075313258	0.04040438	0.105239317
hsa-miR-484	-2.067109609	0.043004837	0.109466857
hsa-miR-497-5p	-3.746255049	0.045305328	0.112759928
hsa-miR-191-5p	1.881203385	0.04843244	0.117922463
hsa-miR-107	2.807810051	0.057381917	0.136332086
hsa-miR-29c-3p	-1.601380481	0.058428037	0.136332086
hsa-miR-148a-3p	-1.845234775	0.062674659	0.143256362
hsa-miR-128-3p	-2.534730071	0.066096263	0.147391478

Table 5.5- Table of top 50 miRNAs that are most differentially expressed by Teffs and Teff EVs.

The list is ranked according to the t-test p-value. FDR means false discovery rate.

5.3.5 Treg EVs contain specific miRNAs that are not found in Teff EVs

As shown in section 4.3.7, human Treg EVs and Teff EVs function differently to affect certain cytokine production, namely Treg EVs inhibited the production levels of IFNγ and IL-6 whereas Teff EVs promoted their production. To understand if these different effects on cytokine production could be due to differences in their miRNA repertoire, the miRNA content between Treg EVs and Teff EVs was compared.

As illustrated in Figure 5.1-D, Treg EVs contain 64 specific miRNAs that are not found in Teff EVs, these miRNAs are listed in Table 5.6 (e.g. miR-146b-3p, miR-125b-5p, miR-369-3p, miR-376b-3p and miR-126-5p). To compare the relative miRNA expression levels between Treg EVs and Teff EVs, a heatmap diagram is displayed (Figure 5.5-A), with the associated volcano plot (Figure 5.5-B). Table 5.7 lists the top 20 miRNAs (e.g. miR-142-3p, miR-150-5p, miR-146a-5p, miR-142-5p and miR-146b-5p) that are expressed higher in Treg EVs compared to Teff EVs whereas Table 5.8 lists the miRNAs that are most differentially expressed between Treg EVs and Teff EVs, ranked according their t-test p-value. To validate the miRNOME finding that Treg EVs expressed higher levels of miR-142-3p and miR-150-5p than in Teff EVs, individual qPCR assays were performed. Treg EVs expressed significantly higher levels of miR-142-3p and miR-150-5p than Teff EVs (Figures 5.5-C and 5.5-D, respectively).

Overall, this data demonstrated that Treg EVs contain exclusive miRNAs and also contain certain miRNAs which are expressed higher in these EVs compared to Teff EVs.

miRNA name	Treg EVs Ct value	Teff EVs Ct value
hsa-miR-99a-5p	>30<40	ND
hsa-miR-331-3p	>30<40	ND
hsa-let-7e-5p	>30<40	ND
hsa-miR-296-5p	>30<40	ND
hsa-miR-34c-5p	>30<40	ND
hsa-miR-146b-3p	>30<40	ND
hsa-miR-141-3p	>30<40	ND
hsa-miR-130b-5p	>30<40	ND
hsa-miR-151a-5p	>30<40	ND
hsa-miR-545-3p	>30<40	ND
hsa-miR-181c-3p	>30<40	ND
hsa-miR-542-5p	>30<40	ND
hsa-miR-125b-5p	>30<40	ND
hsa-miR-501-5p	>30<40	ND
hsa-miR-212-5p	>30<40	ND
hsa-miR-589-5p	>30<40	ND
hsa-miR-200a-3p	>30<40	ND
hsa-let-7b-3p	>30<40	ND
hsa-miR-137	>30<40	ND
hsa-miR-19b-1-5p	>30<40	ND
hsa-miR-744-3p	>30<40	ND
hsa-miR-29b-2-5p	>30<40	ND
hsa-miR-362-5p	>30<40	ND
hsa-miR-378a-5p	>30<40	ND
hsa-miR-34b-3p	>30<40	ND
hsa-miR-124-5p	>30<40	ND
hsa-miR-138-5p	>30<40	ND
hsa-miR-151a-3p	>30<40	ND
hsa-miR-455-5p	>30<40	ND
hsa-miR-340-5p	>30<40	ND
hsa-miR-450b-5p	>30<40	ND
hsa-miR-639	>30<40	ND
hsa-miR-199a-3p	>30<40	ND
hsa-miR-208a-3p	>30<40	ND
hsa-miR-409-3p	>30<40	ND
hsa-miR-1181	>30<40	ND
hsa-miR-493-3p	>30<40	ND
hsa-miR-369-3p	>30<40	ND
hsa-miR-143-3p	>30<40	ND
hsa-miR-145-5p	>30<40	ND
hsa-miR-122-5p	>30<40	ND
hsa-miR-144-5p	>30<40	ND
hsa-miR-616-3p	>30<40	ND
hsa-miR-596	>30<40	ND
hsa-miR-382-5p	>30<40	ND
hsa-miR-139-3p	>30<40	ND
hsa-miR-100-5p	>30<40	ND
hsa-miR-934	>30<40	ND
hsa-miR-885-3p	>30<40	ND
hsa-miR-765	>30<40	ND
hsa-miR-675-3p	>30<40	ND
hsa-miR-638	>30<40	ND
hsa-miR-609	>30<40	ND
hsa-miR-604	>30<40	ND
hsa-miR-584-5p	>30<40	ND
hsa-miR-517-5p	>30<40	ND
hsa-miR-502-5p	>30<40	ND
hsa-miR-376b-3p	>30<40	ND
hsa-miR-34c-3p	>30<40	ND
hsa-miR-218-1-3p	>30<40	ND
hsa-miR-2113	>30<40	ND
hsa-miR-205-5p	>30<40	ND
hsa-miR-133a-3p	>30<40	ND
hsa-miR-126-5p	>30<40	ND

Table 5.6- Table of miRNAs found exclusively in Treg EV and not in Teff EVs.

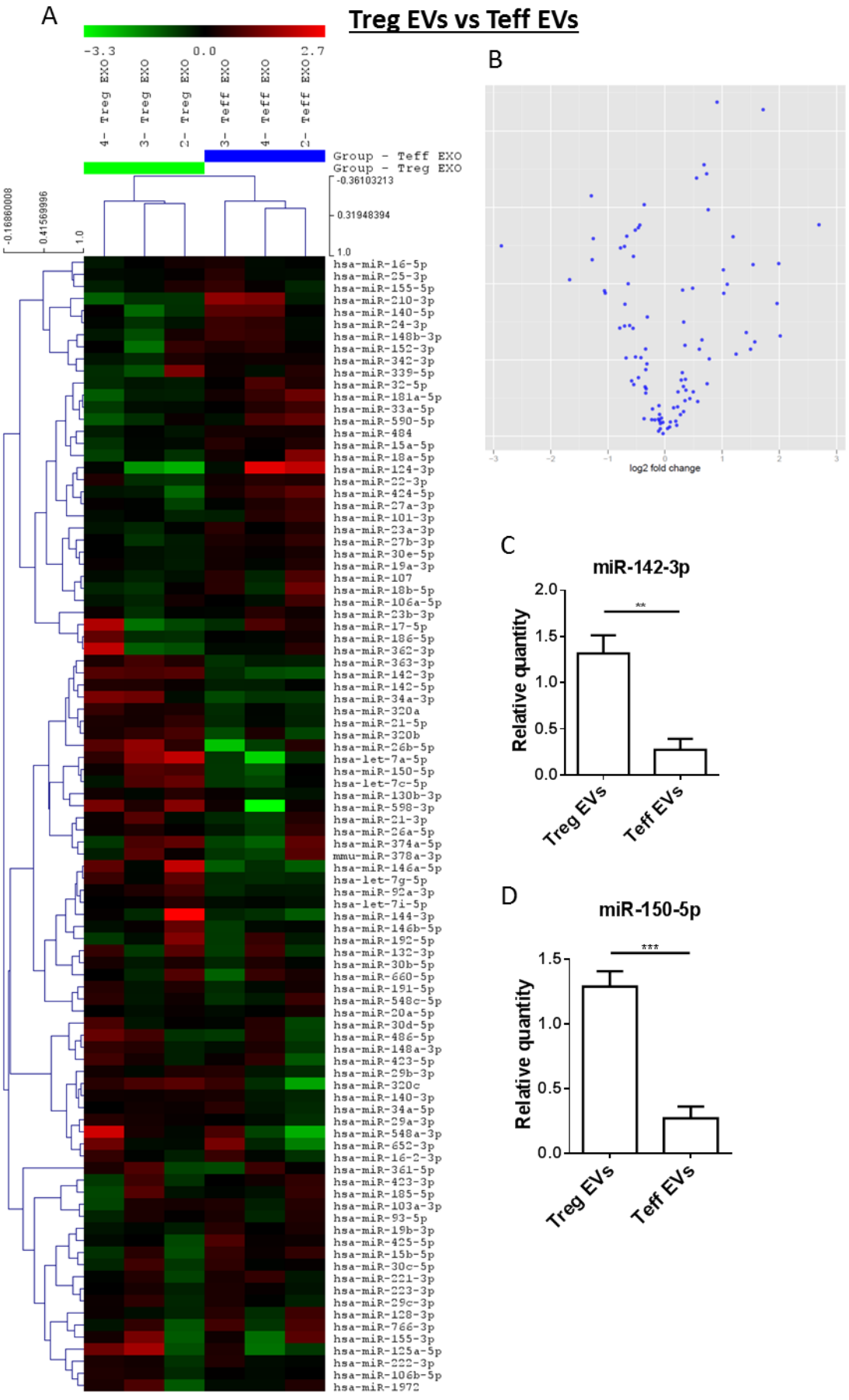

A
Treg EVs vs Teff EVs
-3.3
0.0
2.7
4- Treg EXO
3- Treg EXO
2- Treg EXO
3- Teff EXO
4- Teff EXO
2- Teff EXO
Group - Teff EXO
Group - Treg EXO
-0.36103213
0.31948394
1.0
-0.16860008
0.41569996
1.0
hsa-miR-16-5p
hsa-miR-25-3p
hsa-miR-155-5p
hsa-miR-210-3p
hsa-miR-140-5p
hsa-miR-24-3p
hsa-miR-148b-3p
hsa-miR-152-3p
hsa-miR-342-3p
hsa-miR-339-5p
hsa-miR-32-5p
hsa-miR-181a-5p
hsa-miR-33a-5p
hsa-miR-590-5p
hsa-miR-484
hsa-miR-15a-5p
hsa-miR-18a-5p
hsa-miR-124-3p
hsa-miR-22-3p
hsa-miR-424-5p
hsa-miR-27a-3p
hsa-miR-101-3p
hsa-miR-23a-3p
hsa-miR-27b-3p
hsa-miR-30e-5p
hsa-miR-19a-3p
hsa-miR-107
hsa-miR-18b-5p
hsa-miR-106a-5p
hsa-miR-23b-3p
hsa-miR-17-5p
hsa-miR-186-5p
hsa-miR-362-3p
hsa-miR-363-3p
hsa-miR-142-3p
hsa-miR-142-5p
hsa-miR-34a-3p
hsa-miR-320a
hsa-miR-21-5p
hsa-miR-320b
hsa-miR-26b-5p
hsa-let-7a-5p
hsa-miR-150-5p
hsa-let-7c-5p
hsa-miR-130b-3p
hsa-miR-598-3p
hsa-miR-21-3p
hsa-miR-26a-5p
hsa-miR-374a-5p
mmu-miR-378a-3p
hsa-miR-146a-5p
hsa-let-7g-5p
hsa-miR-92a-3p
hsa-let-7i-5p
hsa-miR-144-3p
hsa-miR-146b-5p
hsa-miR-192-5p
hsa-miR-132-3p
hsa-miR-30b-5p
hsa-miR-660-5p
hsa-miR-191-5p
hsa-miR-548c-5p
hsa-miR-20a-5p
hsa-miR-30d-5p
hsa-miR-486-5p
hsa-miR-148a-3p
hsa-miR-423-5p
hsa-miR-29b-3p
hsa-miR-320c
hsa-miR-140-3p
hsa-miR-34a-5p
hsa-miR-29a-3p
hsa-miR-548a-3p
hsa-miR-652-3p
hsa-miR-16-2-3p
hsa-miR-361-5p
hsa-miR-423-3p
hsa-miR-185-5p
hsa-miR-103a-3p
hsa-miR-93-5p
hsa-miR-19b-3p
hsa-miR-425-5p
hsa-miR-15b-5p
hsa-miR-30c-5p
hsa-miR-221-3p
hsa-miR-223-3p
hsa-miR-29c-3p
hsa-miR-128-3p
hsa-miR-766-3p
hsa-miR-155-3p
hsa-miR-125a-5p
hsa-miR-222-3p
hsa-miR-106b-5p
hsa-miR-1972
B
log2 fold change
C
miR-142-3p
Relative quantity
2.0
1.5
1.0
0.5
0.0
**
Treg EVs
Teff EVs
D
miR-150-5p
Relative quantity
1.5
1.0
0.5
0.0
***
Treg EVs
Teff EVs

Figure 5.5- Treg EVs and Teff EVs contain different miRNA repertoires

(A) *The heatmap diagram shows the two-way unsupervised hierarchical clustering of miRNAs and Treg EVs (first three columns) and Teff EVs (last three columns).* ***(B)*** *Volcano plot displaying the numbers of miRNAs differentially expressed between Treg EVs and Teff EVs. Validation individual qPCR assays showing the expression levels of Treg EVs compared to Teff EVs for* ***(C)*** *miR-142-3p and* ***(D)*** *miR-150-5p. Student t-test was used to test significance. **p<0.01 and ***p<0.001*

miRNA name	Fold change Treg EVs/Teff EVs	t-test p-value	Benjamini-Hochberg FDR
hsa-miR-363-3p	1.87841804	0.006489773	0.30523537
hsa-miR-142-3p	3.28324672	0.007269691	0.30523537
hsa-miR-21-5p	1.603604501	0.016675467	0.30523537
hsa-miR-320a	1.655418014	0.019115478	0.30523537
hsa-miR-142-5p	1.464628332	0.020374887	0.30523537
hsa-miR-92a-3p	1.68683633	0.032835366	0.30523537
hsa-let-7a-5p	6.463770322	0.041200915	0.30523537
hsa-miR-150-5p	2.280412933	0.049357387	0.30523537
hsa-miR-146a-5p	3.965772648	0.073967097	0.321090789
hsa-miR-34a-3p	2.905359936	0.075148908	0.321090789
hsa-let-7g-5p	2.027647471	0.081325904	0.332375435
hsa-let-7c-5p	2.125588792	0.101028546	0.351186397
hsa-miR-29a-3p	1.39700209	0.107213845	0.351186397
hsa-let-7i-5p	1.236812457	0.1103023	0.351186397
hsa-miR-320b	2.037393577	0.11581679	0.351186397
hsa-miR-26b-5p	3.887693478	0.135003942	0.388893403
hsa-miR-130b-3p	1.25331183	0.178597111	0.472975136
hsa-miR-320c	2.675779675	0.209262118	0.491765978
hsa-miR-598-3p	4.039348977	0.220140665	0.504712743
hsa-miR-146b-5p	1.563936878	0.233687797	0.523015547

Table 5.7- Table of top 20 miRNAs found higher in Treg EVs compared to Teff EVs.

The list is ranked according to the t-test p-value. FDR means false discovery rate.

miRNA	Fold change Treg EVs to Teff EVs	t-test p-value	Benjamini-Hochberg FDR
hsa-miR-363-3p	1.87841804	0.006489773	0.30523537
hsa-miR-142-3p	3.28324672	0.007269691	0.30523537
hsa-miR-21-5p	1.603604501	0.016675467	0.30523537
hsa-miR-320a	1.655418014	0.019115478	0.30523537
hsa-miR-142-5p	1.464628332	0.020374887	0.30523537
hsa-miR-181a-5p	-2.445167101	0.026634717	0.30523537
hsa-miR-30e-5p	-1.287984211	0.030457804	0.30523537
hsa-miR-92a-3p	1.68683633	0.032835366	0.30523537
hsa-let-7a-5p	6.463770322	0.041200915	0.30523537
hsa-miR-484	-1.358099651	0.041390939	0.30523537
hsa-miR-19b-3p	-1.382175592	0.043076496	0.30523537
hsa-miR-23a-3p	-1.434745966	0.044690849	0.30523537
hsa-miR-27b-3p	-1.591065514	0.048961515	0.30523537
hsa-miR-150-5p	2.280412933	0.049357387	0.30523537
hsa-miR-424-5p	-2.387755718	0.050746447	0.30523537
hsa-miR-124-3p	-7.273862887	0.05664018	0.30523537
hsa-miR-33a-5p	-1.635344187	0.057155836	0.30523537
hsa-miR-32-5p	-1.715729053	0.058449326	0.30523537
hsa-miR-15a-5p	-1.467263618	0.066367973	0.321090789
hsa-miR-140-5p	-2.419327909	0.069782396	0.321090789
hsa-miR-146a-5p	3.965772648	0.073967097	0.321090789
hsa-miR-34a-3p	2.905359936	0.075148908	0.321090789
hsa-let-7g-5p	2.027647471	0.081325904	0.332375435
hsa-miR-210-3p	-3.181580065	0.094327373	0.351186397
hsa-miR-24-3p	-1.563670237	0.100266279	0.351186397
hsa-let-7c-5p	2.125588792	0.101028546	0.351186397
hsa-miR-29a-3p	1.39700209	0.107213845	0.351186397
hsa-let-7i-5p	1.236812457	0.1103023	0.351186397
hsa-miR-590-5p	-2.084429447	0.111381082	0.351186397
hsa-miR-18a-5p	-2.064673363	0.115443142	0.351186397
hsa-miR-320b	2.037393577	0.11581679	0.351186397
hsa-miR-26b-5p	3.887693478	0.135003942	0.388893403
hsa-miR-425-5p	-1.625768741	0.136526408	0.388893403
hsa-miR-19a-3p	-1.240593093	0.165433357	0.457374574
hsa-miR-130b-3p	1.25331183	0.178597111	0.472975136
hsa-miR-22-3p	-1.536678445	0.188156857	0.472975136
hsa-miR-148b-3p	-1.633670417	0.189487212	0.472975136
hsa-miR-15b-5p	-1.731960688	0.195510919	0.472975136
hsa-miR-27a-3p	-1.47426479	0.196234365	0.472975136
hsa-miR-320c	2.675779675	0.209262118	0.491765978
hsa-miR-598-3p	4.039348977	0.220140665	0.504712743
hsa-miR-146b-5p	1.563936878	0.233687797	0.523015547
hsa-miR-548a-3p	2.968504504	0.240874768	0.526563447
hsa-miR-140-3p	1.273473703	0.253640261	0.537991301
hsa-miR-23b-3p	-1.265608471	0.268195204	0.537991301
hsa-miR-21-3p	1.515030522	0.268947837	0.537991301
hsa-miR-144-3p	2.818905059	0.26899565	0.537991301
hsa-miR-125a-5p	2.365443703	0.289481864	0.562523738
hsa-miR-107	-1.430771155	0.30220773	0.562523738
hsa-miR-101-3p	-1.340155075	0.30573416	0.562523738

Table 5.8- Table of top 50 miRNAs that are most differentially expressed by Treg EVs and Teff EVs.

The list is ranked according to the t-test p-value. FDR means false discovery rate.

5.3.6 Target prediction and pathway analysis of miRNAs enriched in human Treg EVs reveal they are linked to targeting of pro-inflammatory cytokine genes

To understand if miRNAs found exclusively in Treg EVs or found expressed at higher levels in Treg EVs compared their parent Tregs (collectively 'miRNAs enriched in Treg EVs') play a role in modifying target cell functions such as modulating cytokine production as shown in section 4.3.7, a bioinformatics analysis was performed. This bioinformatics tool was available at microRNA.org and was used to predict the miRNA to mRNA interactions in the 3' UTR of IFNγ, IL-2 and IL-6 mRNAs (binding sites with matching seed regions) and to obtain the associated algorithms of mirSVR and PhastCons scores. Figures 5.6-A, B and C, respectively, indicate the full length of the 3' UTR of IFNγ, IL-2 and IL-6 mRNAs showing the binding target locations/sequences of miRNAs enriched in Treg EV. The specific alignment of sequences with seeding sequences and associated mirSVR and PhastCons scores are shown in Appendices 3, 4 and 5, respectively. The mirSVR score measures the miRNA effect on the mRNA expression level with values starting at <0, in other words, the more negative the value the greater the effect (Riffo-Campos, Riquelme, and Brebi-Mieville 2016). The PhastCons scores indicates the conservation of nucleotide sequences between species, the values range from 0 to 1, with a value of 1 being highly conserved (Riffo-Campos, Riquelme, and Brebi-Mieville 2016). As presented in Figures 5.6-A- C, several miRNAs enriched in Treg EVs have one or more targets within each of these pro-inflammatory cytokines 3' UTR of IFNγ, IL-2 and IL-6 mRNAs and notably, multiple miRNAs can target a single cytokine 3' UTR of mRNA. The results of this analysis suggested that miRNAs enriched in Treg EVs could potentially modulate cytokine production in target cells such as Tresps, given that Tresps produce these pro-inflammatory cytokines as shown in Figure 4.7. A summary of the miRNAs that are predicted to target these cytokine mRNAs are shown in Figure 5.6-D, with some miRNAs (miR-369-3p, miR-376c-3p and miR-195-3p) having targets in all cytokine 3' UTR of IFNγ, IL-2 and IL-6 mRNAs analysed.

Given that the miRNOME screen showed that human Treg EVs contained miR-1972, miR-451 and miR-126 at very high levels and 536.20, 311.50 and 327.89 folds higher than in Tregs (Figures 5.3-A and B and Table 5.3), it warranted further investigation. However, this further investigation was limited to bioinformatics analysis and not validated by individual qPCR assays. Nevertheless, the bioinformatics tool, mirTAR

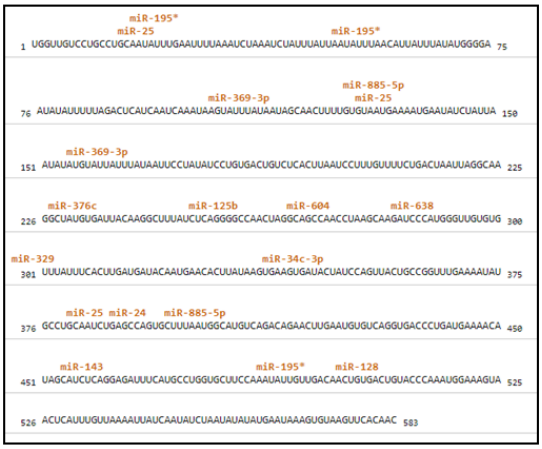
predicted *in silico* binding of miR-1972 to target multiple genes (mRNA) in KEGG pathways related to allograft rejection, graft versus host disease and antigen processing and presentation (Figures 5.7-A-C, respectively), which targeted HLA-A molecules, CD80, CD28 and TNF. KEGG pathways are maps of biological signalling pathways, illustrating genes or molecules involved in particular diseases and conditions (Du et al. 2016).

In silico analysis revealed that miR-451 and miR-126 targets genes involved in the T cell receptor signalling pathways (Figures 5.7-D and E, respectively). As illustrated in Figure 5.7-D, miR-451 targets NFATC1; this is upstream of various cytokine productions pathway, and, miR-126 targets PIK3R2 (Figure 5.7- E), which is immediately downstream of the T cell costimulatory molecules CD28 and ICOS pathways.

Taken together, the prediction targeting of miRNAs enriched in Treg EVs to their target mRNAs suggest that they can inhibit pro-inflammatory cytokines of IFNγ, IL-2 and IL-6 mRNAs from their translation into protein. Furthermore, these miRNAs can possibly inhibit gene expression (mRNAs) associated in KEGG pathways of allograft rejection, graft versus host disease, antigen processing and presentation and T cell receptor signalling pathways. All these observations require further investigation.

A

IFNγ mRNA 3'UTR sequence with relevant miRNA (enriched in Treg EVs) target prediction sites

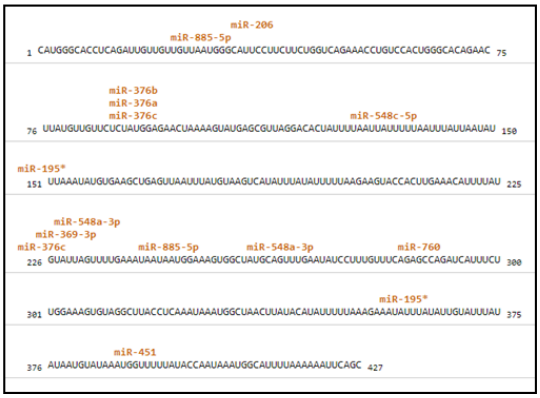


B

IL-2 mRNA 3'UTR sequence with relevant miRNA (enriched in Treg EVs) target prediction sites

C

IL-6 mRNA 3'UTR sequence with relevant miRNA (enriched in Treg EVs) target prediction sites



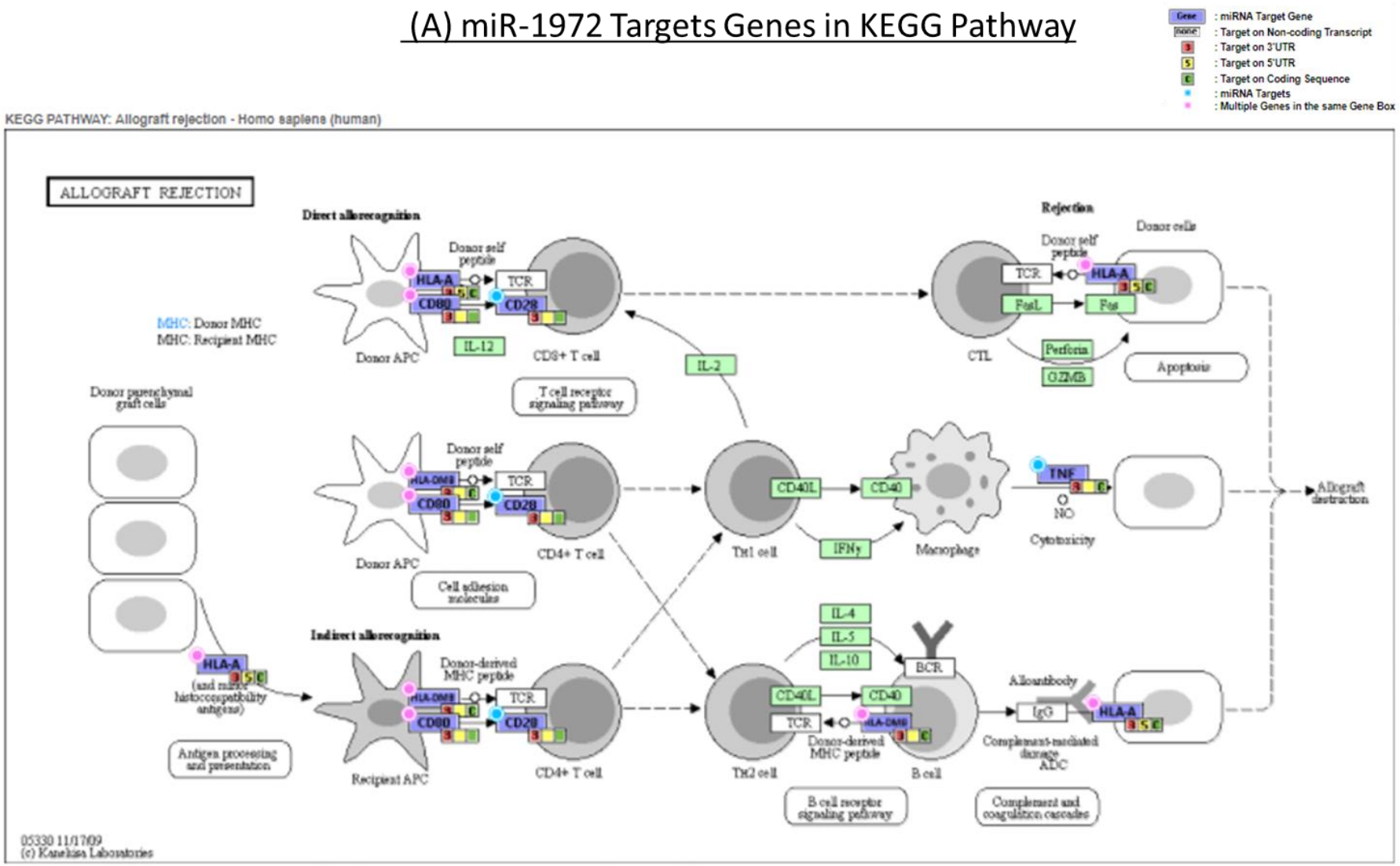
D

miRNA expression in Treg EVs	miRNA enriched in Treg EVs which target relevant cytokine mRNA	miRNA which targets mRNA 3' UTR		
		IFNγ	IL-2	IL-6
Exclusive to Treg EVs	miR-369-3p	✓	✓	✓
Exclusive to Treg EVs	miR-376c-3p	✓	✓	✓
Exclusive to Treg EVs	miR-195-3p	✓	✓	✓
Exclusive to Treg EVs	miR-885-5p	✓	-	✓
Exclusive to Treg EVs	miR-376a-3p	-	✓	✓
Exclusive to Treg EVs	miR-376b-3p	-	✓	✓
Exclusive to Treg EVs	miR-143-3p	✓	-	-
Exclusive to Treg EVs	miR-638	✓	-	-
Exclusive to Treg EVs	miR-604	✓	-	-
Exclusive to Treg EVs	miR-34c-3p	✓	-	-
Exclusive to Treg EVs	miR-329-3p	✓	-	-
Exclusive to Treg EVs	miR-584-5p	-	✓	-
Exclusive to Treg EVs	miR-760	-	-	✓
Exclusive to Treg EVs	miR-206	-	-	✓
Higher in Treg EVs than Tregs	miR-25-3p	✓	-	-
Higher in Treg EVs than Tregs	miR-128-3p	✓	-	-
Higher in Treg EVs than Tregs	miR-24-3p	✓	-	-
Higher in Treg EVs than Tregs	miR-125b-5p	✓	-	-
Higher in Treg EVs than Tregs	miR-451a	-	-	✓
Higher in Treg EVs than Tregs	miR-548c-5p	-	-	✓
Higher in Treg EVs than Tregs	miR-548a-3p	-	-	✓

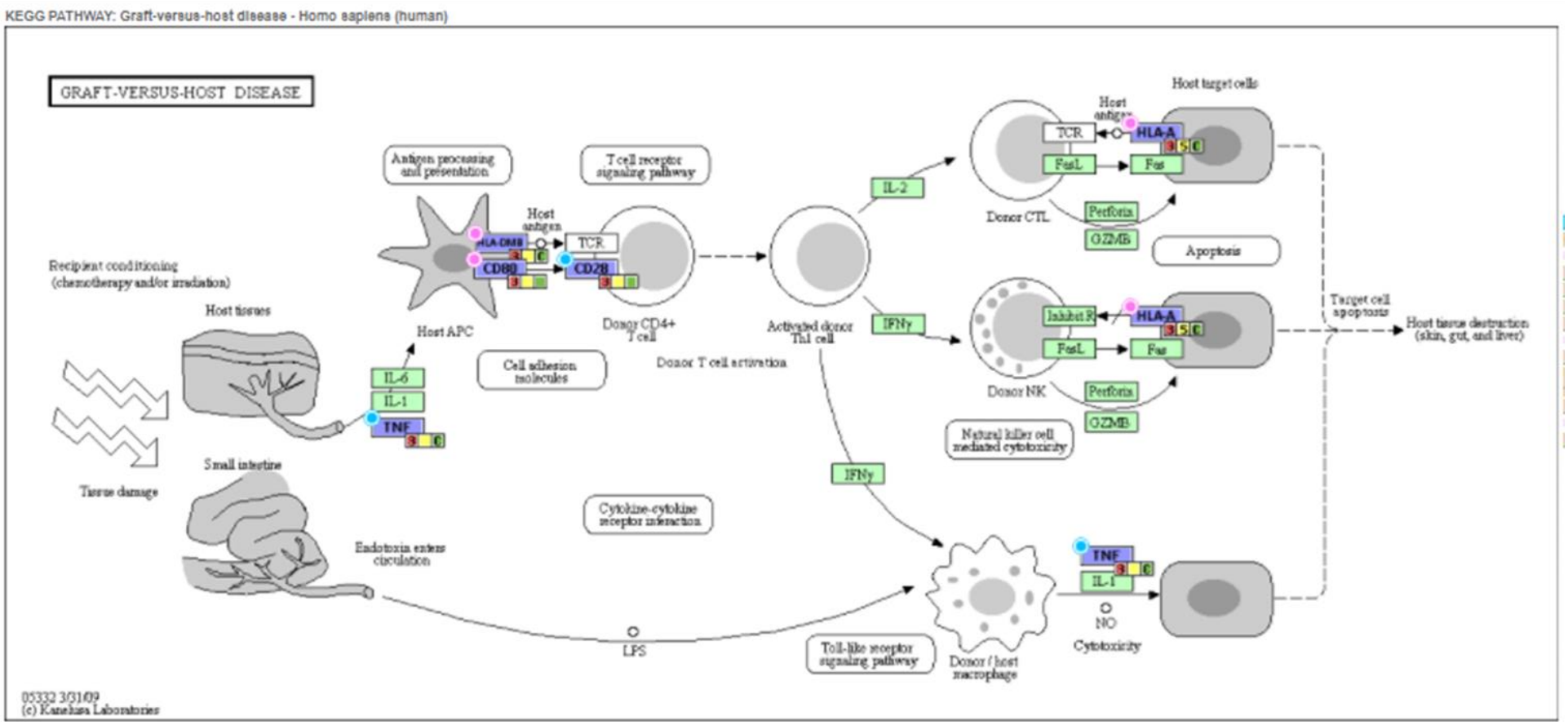
Figure 5.6- Target prediction of miRNAs enriched in human Treg EVs reveals they are linked to targeting of pro-inflammatory cytokine genes

3' UTR mRNA sections are displayed with the predicted targeting sites of various miRNAs found enriched in Treg EVs **(A)** *IFNγ* **(B)** *IL-2 and* **(C)** *IL-6.* **(D)** *Summary of miRNAs that are enriched in Treg EVs and also had targeting prediction to 3' UTR of IFNγ, IL-2 and/or IL-6 mRNA.*

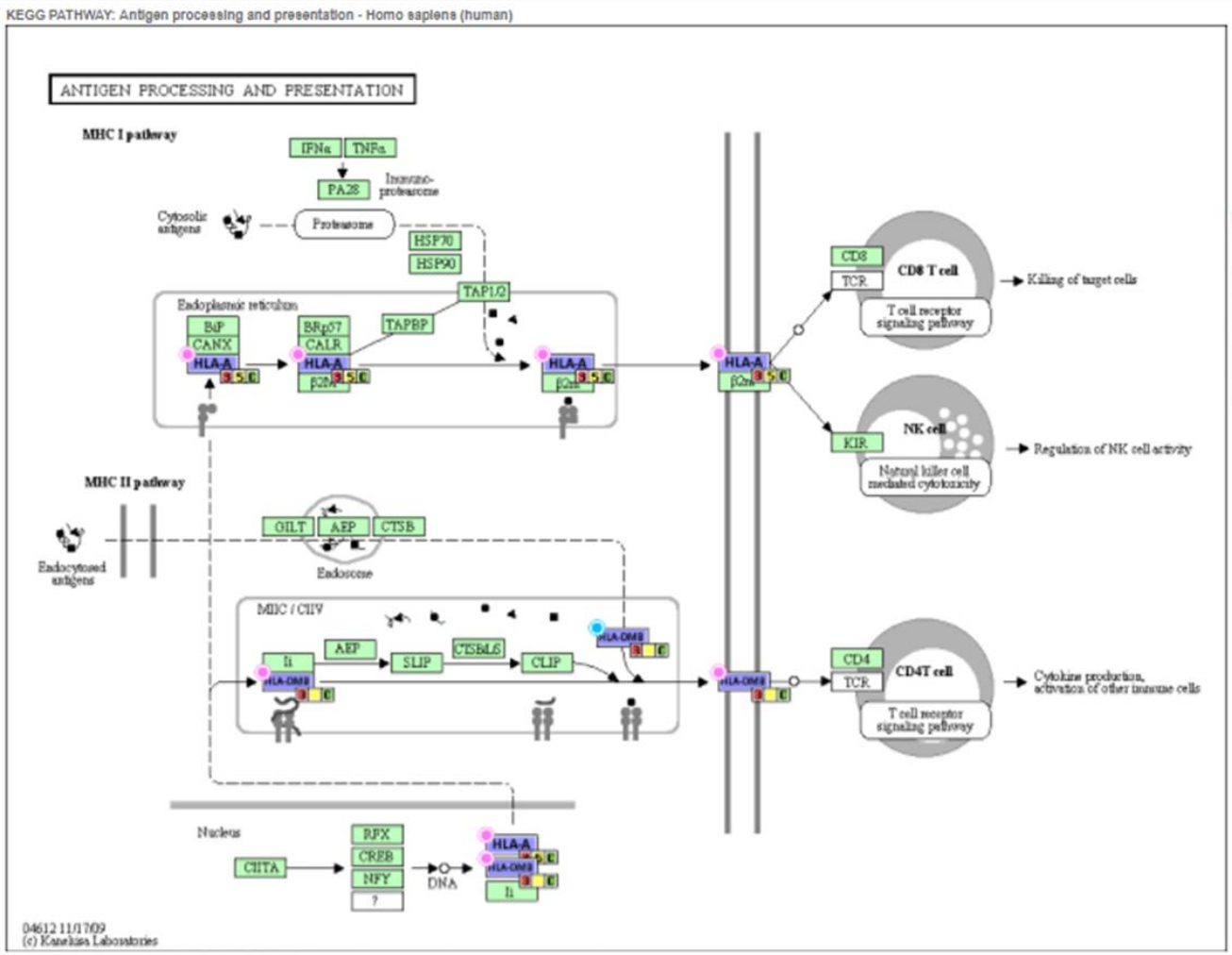
(A) miR-1972 Targets Genes in KEGG Pathway



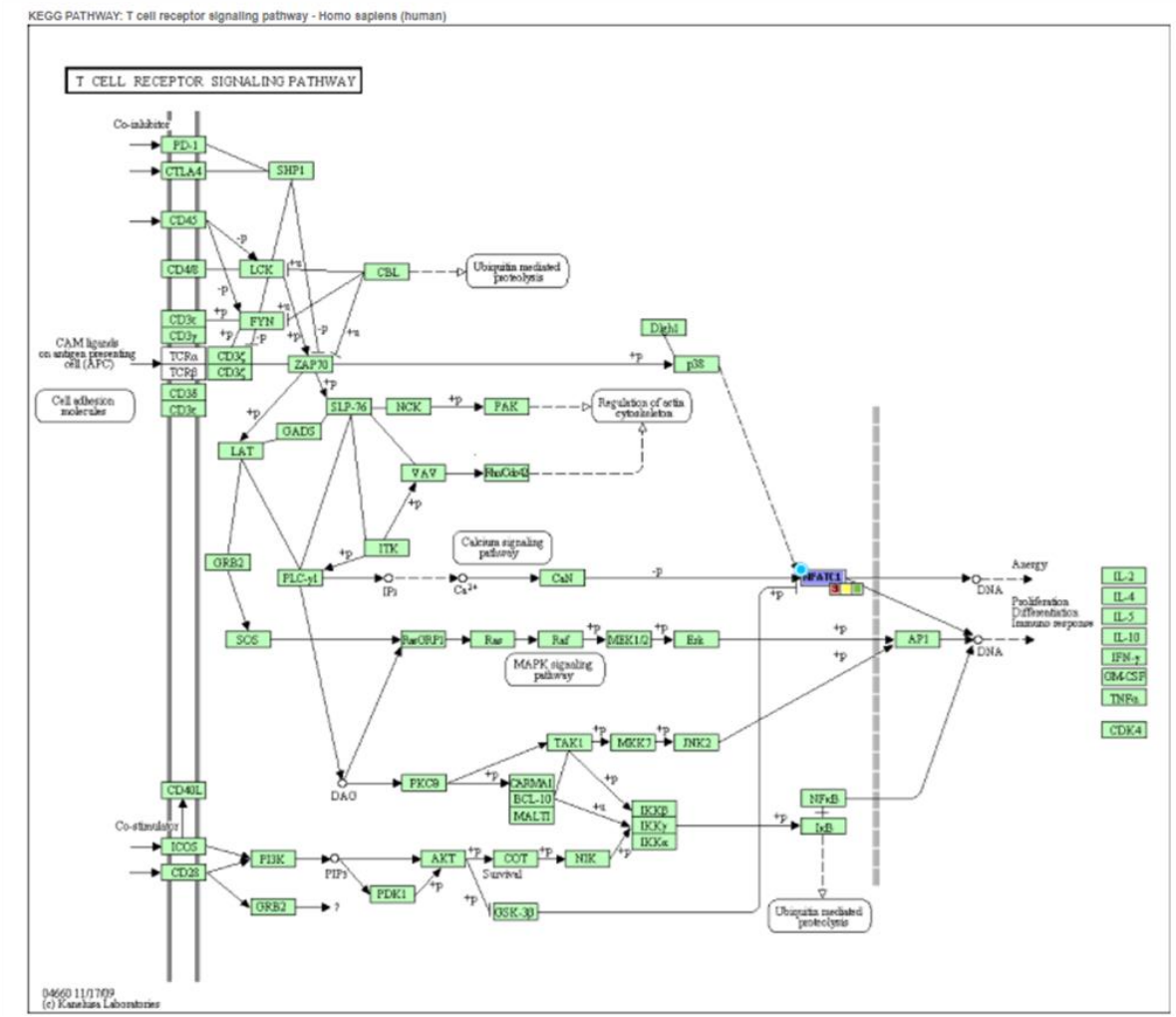
(B) miR-1972 Targets Genes in KEGG Pathway



(C) miR-1972 Targets Genes in KEGG Pathway



D) miR-451 Targets Genes in KEGG Pathway



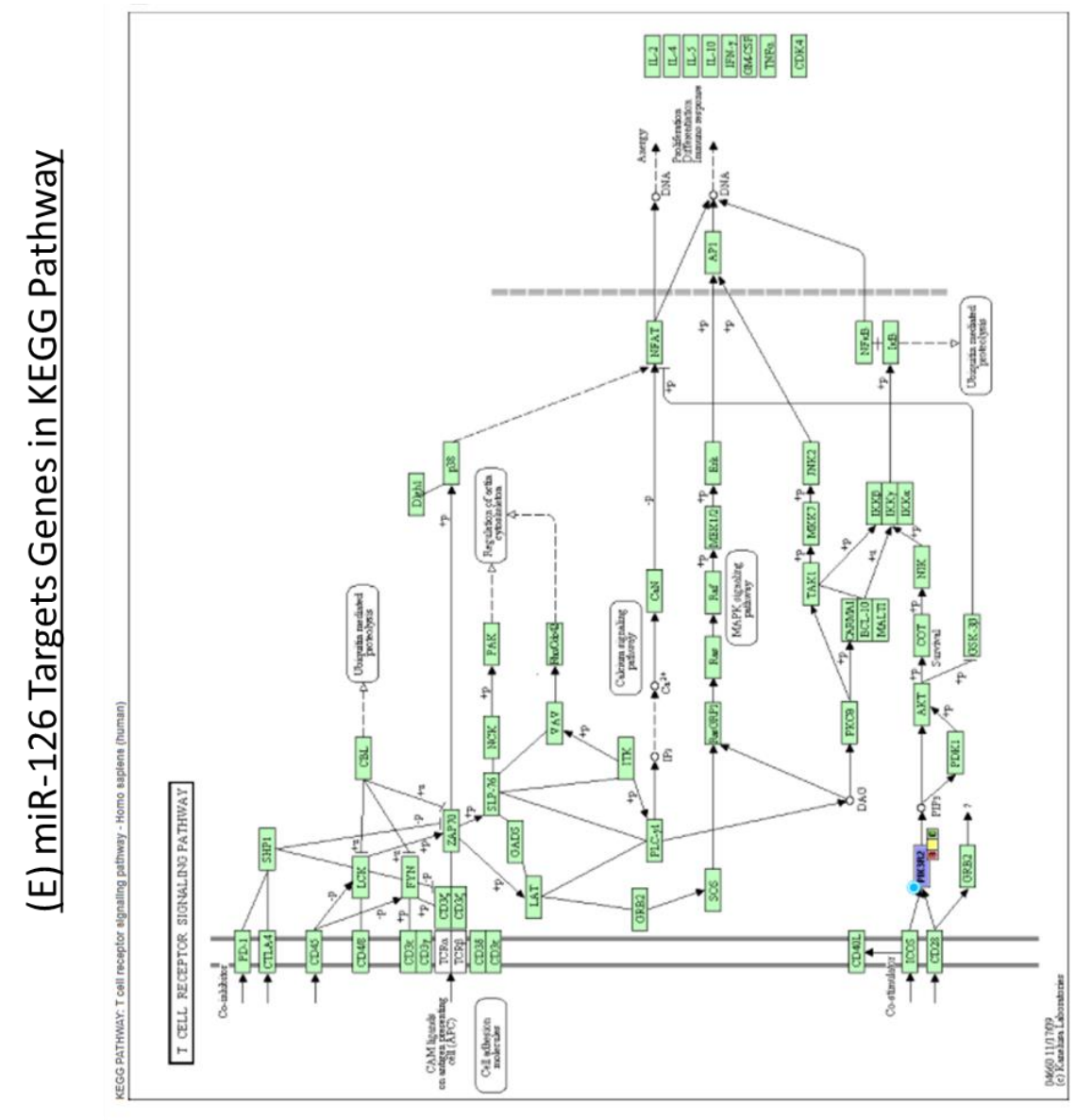


Figure 5.7- Bioinformatics analysis of miR-1972, miR-451 and miR-126.

Pathway analysis of miRNAs that are highly enriched in Treg EVs predicted to target various genes involved in the immune system pathway. miR-1972, miR-451 and miR-126, which are highly enriched in human Treg EVs, reveal they are linked to allograft rejection and T cell receptor signalling KEGG pathways. Pathway analysis of miR-1972 predicted that it targets multiple genes involved in ***(A)*** *Allograft rejection,* ***(B)*** *GvHD* ***(C)*** *Antigen processing and presentation. Pathway analysis of* ***(D)*** *miR-451 and* ***(E)*** *miR-126 predicted that it targets genes involved in the T cell receptor signalling pathways.*

5.4 Discussion

As above explained, during my PhD studies, another group had already published that human Tregs release EVs that contain miRNAs (Torri et al. 2017), but what is unknown is whether human Treg EV-associated miRNAs can modulate cytokine production by target cells such as Tresps. As shown in section 4.3.7, human Treg EVs inhibited the production levels of IFNγ, IL-2 and IL-6 by Tresps. In this chapter, I have shown that Treg EVs contain a collection of miRNAs that are exclusive or found at higher expression levels compared to their parent Tregs or compared to Teff EVs, which indicates that these miRNAs may be specifically packaged into Treg EVs. The bioinformatics analysis revealed that some of the miRNAs found enriched in Treg EVs, namely miR-369-3p, miR-376c-3p and miR-195-3p were amongst other miRNAs that directly target the 3' UTR of IFNγ, IL-2 and IL-6 mRNA, therefore these miRNAs have the potential to inhibit the production of these cytokines, which may in part explain the observations shown in section 4.3.7. Treg EVs harboured over 300 folds higher expression levels of miR-1972, miR-451 and miR-126 that in their parent Tregs; bioinformatics analysis unravelled that these miRNAs target signalling pathways related to allograft rejection, graft versus host disease, antigen processing and presentation and T cell receptor signalling.

However, it is noteworthy that Treg EVs contain other miRNAs that are common to their parent Tregs, but even at lower expression levels than Tregs, it may still contribute to modulating target cell functions. The analysis performed herein were on miRNAs that were enriched in Treg EVs (either found exclusively or found higher expressed than Tregs), but other miRNAs found at lower expression levels than the Tregs cannot be ruled out for not affecting cytokine production or other target cell function modulation.

When the cells were compared against EV-associated miRNAs, of the top 50 miRNAs, the EVs were more similar to each other than to their parent cells, in agreement with a previous report suggesting that EVs derived from various cells are more similar to each other than their parent cells (Mittelbrunn et al. 2011). Given that EVs package their miRNA in a specific manner, it is therefore reasonable to observe that these Treg EVs and Teff EVs may have some commonly expressed miRNAs. The packaging of miRNA cargo into EVs is a regulated process with a sorting mechanism in place to select specific miRNAs to be packaged into EVs which was demonstrated by Villarroya-Beltri *et al.*

(Villarroya-Beltri, Gutiérrez-Vázquez, Sánchez-Cabo, et al. 2013). The authors showed that sumoylated hnRNPA2B1 controls which miRNAs are packaged into exosomes through its interaction with specific motifs (Villarroya-Beltri, Gutiérrez-Vázquez, Sánchez-Cabo, et al. 2013). Other mechanisms of miRNA sorting into EVs may also exist. Of note, parental T cells and their EVs-contained mRNA and miRNA repertoire differ, indicating a possible regulated specific sorting of RNA molecules into EVs for targeted roles (Gutiérrez-Vázquez et al. 2013).

5.4.1 miR-142-3p, miR-150-5p, Let-7d

As shown in section 3.3.8, mouse Treg EVs contain higher expression levels of miR-142-3p and miR-150-5p compared to FoxP3^{low} T cell EVs. Interestingly, human Teffs had higher expression levels of miR-142-3p and miR-150-5p than in Tregs, although Treg EVs contained higher expression levels of miR-142-3p and miR-150-5p than in Teff EVs. This indicates that Tregs may have specifically packaged a higher amount of miR-142-3p and miR-150-5p molecules as these miRNAs have inhibitory roles and thus when delivered to target cells can suppress their cellular functions. As I had already discussed in section 3.4, miR-142-3p and miR-150-5p have been shown by previous studies to be involved in inhibiting the immune response, and thus the roles of these miRNAs will not be discussed again in this thesis chapter, but I would refer the reader to section 3.4. I also found the expression of Let-7d in human Treg EVs, in line with Okoye *et al*. Okoye *et al*. described that mouse Treg EVs contained Let-7d, which played a role in the reduction of IL-2 and IFNγ production by target cells (Okoye et al. 2014). Similarly, these human counterparts could be utilising Let-7d in a similar manner.

5.4.2 miRNAs that target IFNγ, IL-2 and IL-6

Bioinformatics analysis *in silico* predicted miRNAs enriched in Treg EVs to target pro-inflammatory IFNγ, IL-2 and IL-6 cytokines mRNAs within the 3' UTR from translating into protein. As demonstrated in section 4.3.7, Treg EVs can significantly inhibit the production levels of IFNγ, IL-2 and IL-6 by Tresps. Indeed, this has been shown in the mouse Treg setting as mouse Treg exosomes can induce alterations in target cell production levels of cytokines as shown in section 3.3.11 and by others (Lesley Ann

Smyth et al. 2013; Okoye et al. 2014), owing to miRNA carried by exosomes (Okoye et al. 2014). Torri *et al*. found that human T helper cell subsets, Th1, Th17 and Treg cells released EVs which had a different miRNA repertoire. These authors showed that Treg EVs but not Th1 EVs nor Th17 EVs inhibited $CD4^+$ T cell proliferation. Furthermore, these Treg EVs were highly enriched in miR-146a-5p, which suppressed Stat1 and Irak2 in target cells (Torri et al. 2017). In agreement to this PhD thesis chapter, human Treg EVs used in this project also contained miR-146a-5p which was 3.96 folds higher in expression levels than in Teff EVs. This was also the trend for the expression levels of miR-146a-5p in Tregs at 3.17 folds higher than in Teffs. This suggests that miR-146a-5p may possibly play an important role of regulating the function of target cells. However, even though similar miRNAs were found in both Torri *et al*.'s paper (Torri et al. 2017) and in this thesis chapter, there are discrepancies which may be due to different EV populations isolated and thus different miRNA repertoires.

Human Treg EVs held a collection of miRNAs which were found exclusive or higher expressed than in their parent Tregs. Of note, certain miRNAs enriched in Treg EVs; miR-369-3p, miR-376c-3p (also named as miR-368 in literature) and miR-195-3p all had bioinformatics predicted targets in 3' UTR for IFNγ, IL-2 and IL-6 cytokine mRNAs. These miRNAs have been reported in literature to affect various cell types. For example, miR-195 has been shown to inhibit macrophage pro-inflammatory profile and polarising their phenotype, in particular IL-6 was significantly decreased by miR-195 (Bras et al. 2017), thus experimentally validating this miRNA target of IL-6 protein output. Indeed, the host laboratory have recently published that Tregs inhibit macrophage production of IL-6 (Romano et al. 2018). Although speculative, this could have been mediated by Treg EVs transporting this miRNA into the macrophages leading to reduced production of IL-6 and warrants further investigation.

As shown in section 4.3.7, human Treg EVs and Teff EVs affect cytokine production by Tresps differently. Treg EVs suppressed the production levels of IL-6, whereas Teff EVs increased this. Both miR-142-3p and Let-7g-5p were found at higher expression levels in Treg EVs compared to Teff EVs. These miRNAs were experimentally demonstrated by Huang *et al*. to both negatively regulate IL-6 production in leukocytes (Huang et al. 2017), this finding further supports the notion that Treg EVs cargo are packaged with specific roles in the target cells. Although the bioinformatics analysis did not highlight

miR-142-3p and Let-7g-5p to target IL-6, this might due to the sensitivity and settings used in the bioinformatics program. miR-125-5p is a miRNA that was commonly expressed by all the samples tested but were found at higher levels in Treg EVs compared to Teff EVs. GATA3 stabilises FoxP3 expression to prevent Tregs differentiating into inflammatory cells. Furthermore, GATA3 induces miR-125a-5p expression to target IL-6R and STAT3 mRNA. By using miR-125a-5p it dampens the sensitivity of Tregs toward IL-6 mediated conversion (Huang et al. 2017). This adds to the notion that Treg EVs may be skewing the T cells towards increasing their FoxP3 expression to differentiate them into induced Tregs. The over-expression of miR-376c-3p on human adenocarcinoma cell line promoted cell apoptosis and suppressed tumour growth *in vivo* (Tu et al. 2016). Whether Treg EVs promoted target cell apoptosis was not tested but given that Tregs use perforin/granzyme as a means of immunosuppression (section 1.6.3.3), it is therefore plausible that EVs derived from Tregs may also harbour this ability.

5.4.3 miR-1972, miR-126-3p and miR-451

miR-1972, miR-126-3p and miR-451 that were found >300 folds higher in Treg EVs compared to their parent Tregs have also been reported in literature to affect various cell types. For example, Agatheeswaran *et al*. demonstrated that miR-1972 induces G2-M cell cycle arrest in KCL22 cancer cells (Agatheeswaran, Pattnayak, and Chakraborty 2016), suggesting that this miRNA may also affect Tresps in a similar manner, although cell cycle arrest was not directly tested in this PhD project, I did however test Tresps proliferation and observed that Treg EVs have the ability to significantly suppress their proliferation which relates to cell cycle arrest. Whether in this project, the suppression of Tresps proliferation was due to the actions of miR-1972 initiating cell cycle arrest as shown by Agatheeswaran *et al*. (Agatheeswaran, Pattnayak, and Chakraborty 2016) was not tested experimentally, but *in silico* prediction analysis revealed that miR-1972 can target HLA-A molecules, CD28, CD80 and TNF. As demonstrated by Appleman *et al*., CD28 costimulation directly regulates T cell cycle entry and the progression into the G1 phase of cell cycle (Appleman et al. 2000). Hence, miR-1972 found contained in Treg EVs have a potential to inhibit CD28 costimulation in Tresps which may lead to their cell

cycle arrest and thus provide an explanation for Treg EVs' ability to inhibit Tresps proliferation.

Wu *et al*. demonstrated that miR-126-3p targets PIK3R2 resulting in suppressed cell proliferation, arrested cell cycle progression and cell apoptosis in sarcoma cells (X.-J. Wu et al. 2016). Similarly, Xiao *et al*. found that overexpression of miR-126 in bladder cancer cells inhibits proliferation, migration, promoted cell apoptosis and induced S cell cycle phase arrest which was due to regulation of the PI3K/Akt signalling pathway (J. Xiao et al. 2016). As shown in Figure 5.7-E, miR-126-3p targets PIK3R2 within the TCR signalling pathway. PIK3R2 is immediately downstream of CD28 signalling. Furthermore, the PIK3R2, which is also known as P85β, is a subunit of PI3K and thus regulates PI3K enzyme activity. PI3K/AKT/mTOR pathway is essential for T cell development and activation. Various studies have shown that blocking the PI3K/AKT/mTOR pathway inhibits T cell activation (Herrero-Sánchez et al. 2016; Xue et al. 2008). Chu *et al*. recently published that miR-126 deficiency enhanced the activation and function of $CD4^+$ T cells (Chu et al. 2018). Although, not shown directly, it is probable that the reverse of increasing miR-126 expression decreases $CD4^+$ T cell activation and function which further supports the above findings. Furthermore, miR-126 was shown to be important for the suppressive ability of Tregs and silencing miR-126 results in reduced FoxP3 expression by targeting of PIK3R2 (Qin et al. 2013). Therefore, it is tempting to speculate that Treg EVs specifically packaged over 300 folds higher expression levels than their Tregs of miR-1972 and miR-126-3p in an intended way to suppress Tresps activation and proliferation given their targeting of CD28 and PIK3R2.

In 2017, Chapman *et al*. demonstrated that miR-451 limited $CD4^+$ T cell proliferation in response to infection in mice. These authors showed that Myc that has a role in cell cycle progression and cell proliferation was a target of miR-451 (Chapman et al. 2017). Additionally, Zeng *et al*. demonstrated that T cells transfected with a miR-451a mimic inhibited their activation and proliferation by targeting Myc (Zeng et al. 2017). Taken together, miR-451 may also play an important role in suppressing T activation and proliferation. Furthermore, miR-451 is linked to DC cytokine regulation of IL-6 (Rosenberger et al. 2012), which supports the bioinformatics analysis finding that miR-451 targets IL-6 mRNA 3' UTR.

5.4.4 miRNAs found in human Treg EVs that are linked to inducing or promoting Tregs

From the human miRNOME results, miR-10a-3p was found in Treg EVs. Bluestone's group published a paper demonstrating how miR-10a marks Tregs due to miR-10a's contribution to stabilise mouse Tregs via FoxP3 expression (Jeker et al. 2012). Whether in the human setting, miR-10a is essential for FoxP3 expression in human Tregs is unclear. However, given that in the human miRNOME, I found miR-10a-3p in human Treg EVs but not in human Tregs might suggest that other miRNAs may be in place to stabilise FoxP3 in human Tregs. One of these other miRNAs could be miR-21. miR-21-5p expression levels was higher in human Treg EVs compared to Teff EVs. As demonstrated by Rouas *et al*., miR-21 in an indirect manner positively regulates FoxP3 expression, whereas, on the other hand, miR-31 negatively regulates FoxP3 expression in human Tregs (Rouas et al. 2009).

In 2014, Dong *et al*. found that in rheumatoid patients' Tregs had a significant decrease of miR-21 and FoxP3 mRNA expressions compared to healthy controls, which was associated with increased STAT3 and decreased STAT5 protein expression and a skewing towards Th17 cells (Dong et al. 2014). Due to these findings, it was suggested that miR-21 may be part of a negative feedback loop dysregulated in rheumatoid arthritis patients which might explain the imbalance between Th17 cells and Tregs. Indeed, the study by Rouas *et al*. supports this hypothesis (Rouas et al. 2009). IL-2 signalling is crucial for Treg differentiation and can be more efficient by miRNA-driven downregulation of the IL-2R signalling inhibitors of SOCS1 and STAT3. These inhibitors keep STAT5 transcription factor inactive, however STAT5 is involved in many aspects of Treg activity and important for their FoxP3 expression (Passerini et al. 2008). miR-155 have been demonstrated to target SOCS1 (Ye et al. 2016; Pathak et al. 2015; D. Wang et al. 2018) and miR-21 to target STAT3 (Hippen et al. 2018; Haider et al. 2010). Together, this suggest that miR-21 found in Treg EVs could be targeting STAT3 and thus promoting STAT5 signalling and promoting FoxP3 expression, possibly to convert Tresps into induced Tregs. For instance, from the human miRNOME, miR-155 was commonly expressed in all the sample types of Tregs, Teffs, Treg EVs and Teffs EVs. Increased expression levels of miR-155 enhances the inhibitory function of $CD39^{+}$ Tregs (J. Liu et al. 2015). Additionally, FoxP3 directly induces miR-155 and promotes the survival of Tregs through suppressing SOCS1 (L.-F. Lu et al. 2009; Kohlhaas et al. 2009).

It is important to note that the mentioned publications reflects the miRNAs that are found exclusively or higher expressed in Treg EVs compared to Tregs or Teff EVs, but as Treg EVs will contain other common miRNAs to Tregs and Teff EVs (but at the same or lower expression levels), these common miRNAs may still contribute to immunosuppression given that Tregs and Teff EVs (and Teffs) were all immunosuppressive (sections 4.3.3 and 4.3.4). However, as a step towards unravelling the complex miRNA repertoire, the miRNAs found exclusively or higher expressed in Treg EVs compared to Tregs or Teff EVs was used.

5.5 Summary

Taken together, the miRNAs found in Treg EVs are likely to play a role in modifying the *in vitro* immune responses observed in Chapter 4 and warrants further validation. Specifically, human Treg EVs significantly inhibited the production levels of IFNγ, IL-2 and IL-6 by Tresps which may be driven by a collection of miRNAs found exclusively or highly enriched in Treg EVs. Bioinformatics analysis predicted these miRNAs to target the 3' UTR the pro-inflammatory cytokines; IFNγ, IL-2 and IL-6 mRNA, hence inhibiting their translating into protein and to play a role in suppressing genes within the allograft rejection pathway (KEGG pathway).

Chapter 6

Assessing Human Treg EV functions *in vivo*

Chapter 6 - Assessing Human Treg EV functions *in vivo*

6.1 Introduction

In this thesis chapter I will present data that assesses the function of Treg EVs *in vivo*. To address this aim, humanised mouse models were used. To understand whether Treg EVs have the potential to reach the transplanted human skin allograft, the expression of homing receptors and the trafficking of EVs to the allograft were tested.

6.1.1 Humanised mouse models of transplantation

The results obtained by manipulating the immune system of rodents have not been translated into the clinical setting and this is due in part to the fundamental differences that exist between animal and human cells and their immune responses (Mestas and Hughes 2004; Kenney et al. 2016). An example of this is when mouse tissues/organs have been depleted of $CD45^+$ cells before transplantation; it significantly promotes the longevity of the graft and in some cases induces immune tolerance. On the contrary, the removal of $CD45^+$ cells in human tissues provides no advantage as the graft still rapidly rejects (Mestas and Hughes 2004; Wood 2003). Another key example is the non-obese diabetes (NOD) mice which spontaneously develop diabetes (Makino et al. 1980), but when treated with rapamycin and IL-2 prevented spontaneous and recurrent autoimmune diabetes following islet transplantation (Rabinovitch et al. 2002). However, when this approach was used in a clinical trial, rapamycin and IL-2 treatment caused a detrimental outcome in humans as this treatment impaired β-cell function (Long et al. 2012).

Thus, the development of immunodeficient mice for engrafting and therefore reconstituting the mice with a functional human immune system have been extensively researched (Kenney et al. 2016; Safinia et al. 2016). To achieve engraftment in mice, it is vital to use immunocompromised mice as the transfer of human cells would otherwise be targeted for destruction by xeno-immune responses mediated by the existing mouse T cells (Kenney et al. 2016). To date, there are various strains of immunodeficient mice that can be used for the engraftment of human haematopoietic cells, the common strains used are: NOD.Cg-*Prkdc*^{scid}*Il2rg*^{gtm1Wjl} (NSG), NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Sug} (NOG),

NOD.Cg-*Rag1*^{tm1MoM}*Il2rg*^{tm1Wjl} (NRG) and C.Cg-*Rag2*^{tm1Fwa}*Il2rg*^{tm1Sug} (BRG). NSG, NOG and NRG are all NOD strains of mice and lack T, B and NK cells with additional defects in innate immune cells (Kenney et al. 2016). Whereas, BRG mice has a mixed genetic background mainly derived from the BALB/c strain, nonetheless BRG mice also lack T, B and NK cells but the remaining mouse innate immune cells are functional (Kenney et al. 2016).

The NSG and NRG mice have mutations in IL-2Rγ which means there are no expressions of these genes and thus without IL-2Rγ, it will not bind cytokines. NOG mice lack the intracytoplasmic domain of IL-2Rγ which means these cells will recognise cytokines but will not transduce downstream signalling. BRG mice lack the intracytoplasmic domain of IL-2Rγ also (Kenney et al. 2016). This receptor binds to cytokines but cannot signal and thus these mice are unresponsive to IL-2, IL-4, IL-9, IL-15, IL-17 and IL-21. Blocking these cytokines signalling pathways effectively inhibits innate and adaptive immunity (Kenney et al. 2016). These mice lack RAG2 and consequently, the rearrangement of TCR/BCR loci is inhibited and thus BRG mice lack functional T and B cells and NK cells. However, as described above, the remaining mouse innate immune cells are functional (Kenney et al. 2016), these functional granulocytes can significantly damage transplanted human tissues hence to achieve high levels of human cell engraftment, continual depletion of these granulocytes are required and can be performed through twice weekly injections of mouse anti-granulocyte receptor 1 (anti-Gr1) antibody (Racki et al. 2010).

The use of humanised mouse models as pre-clinical models for transplantation research, in particular with transplant rejection have undoubtedly been instrumental in advancing the understanding of the human immune system (Issa et al. 2010; Safinia et al. 2016; Kenney et al. 2016). Various human tissues can be transplanted into mice; the most frequently used human tissue for transplantation onto mice is human skin as it is readily available from surgical procedures and due to the less invasive procedure of transplantation onto mice with this tissue type.

The host laboratory has already established a humanised mouse model of transplantation using BRG mice and using human skin (P. Sagoo et al. 2011), whereby the transfer of allogeneic PBMCs causes alloimmune mediated graft damage. This damage was measured by inflammation of the epidermal layers and destruction of vascular/vessel structures, which correlated with human cell intragraft infiltration (P.

Sagoo et al. 2011). However, this level of alloimmune mediated damage was prevented with the additional adoptive transfer of alloantigen specific Tregs, which were more potent at inhibiting the inflammatory response compared to polyclonal Tregs (P. Sagoo et al. 2011; Putnam et al. 2013). The BRG strain of mice were selected as the humanised mouse models of human skin transplantation to test the *in vivo* function of Treg EVs in this PhD thesis.

6.1.2 Homing receptors and tracking EVs *in vivo*

An important *in vivo* feature of Tregs is their ability to migrate to specific locations to mediate efficient immune responses (Issa et al. 2012). Trafficking of Tregs is principally driven by homing receptors, chemokine to chemokine receptors and integrin to integrin ligands interactions (Lim, Broxmeyer, and Kim 2006; S. Wei, Kryczek, and Zou 2006).

CD62L is an important homing receptor found on Tregs (Lim, Broxmeyer, and Kim 2006; S. Wei, Kryczek, and Zou 2006). In previous studies, murine $CD62L^+$ Tregs and $CD62L^-$ Tregs were both able to suppress T cell activation (Szanya et al. 2002; A M Thornton and Shevach 2000), however the $CD62L^+$ Tregs rather than the $CD62L^-$ Tregs were more efficient in protecting against lethal acute GvHD (Ermann et al. 2005; Taylor et al. 2004) and delayed diabetes onset in NOD mice (Szanya et al. 2002). Furthermore, blocking CD62L activity with neutralising antibodies blocked Treg expansion in draining lymph nodes and led to rapid cardiac allograft rejection (Ochando et al. 2005). Thus, entry into the secondary lymphoid organs is important and made efficient by the expression of CD62L on Tregs.

CCR4 is a skin homing receptor found on Tregs (Issa et al. 2012). CCR4 expression on Tregs allows for their migration toward its ligands CCL17 and CCL22, which are produced by mature dendritic cells, thus mediating migration of Tregs into lymphoid tissues (Halim et al. 2017).

Tracking of EVs *in vivo* and understanding their migratory patterns and how they are distributed *in vivo* can aid their development as potential delivery vehicles. Takahashi *et al*. provided some insights into the trafficking of exosomes *in vivo*, these authors showed that exosomes can be labelled with Gaussia luciferase-lactadherin fusion

protein and visualised using whole body imaging was possible and tracking can be performed for hours (Y. Takahashi et al. 2013).

Labelling of exosomes can also be performed by transfecting the parent cells with CD63-eGFP (Mittelbrunn et al. 2011) and visualised *in vivo* (Lai et al. 2015) and using whole body optical imaging (M. Yang et al. 2000). Tracking EVs in the context of a humanised mouse model of transplantation will provide invaluable insights into the spatiotemporal dynamics which may aid the understanding of their specific immunomodulatory functions. However, the migratory route of Treg EVs post-injection into an animal host remains elusive, but important to understand if used as a potential immunotherapeutic agent.

6.2 Aims and objectives

Aim

The aim of this thesis chapter was to assess whether Treg EVs function *in vivo* by assessing their ability to suppress alloimmune mediated skin allograft damage.

Objectives:

(1) Test for the presence of homing receptors on Tregs and their EVs to provide an understanding of their potential migratory ability.
(2) Assess the suppressive function of Treg EVs and Teff EVs *in vivo* using humanised mouse models of human skin transplantation.
(3) To visualise Treg EVs *in situ* by labelling of Treg EVs using eGFP-CD63 and mCherry-CD63.

6.3 Results

6.3.1 Human Tregs, Teffs and Treg EVs express homing receptors

As previously described, homing of Tregs to specific locations is important for their efficient function (Issa et al. 2012). To test that the Tregs and Teffs express homing markers, Tregs and Teffs were immunostained with antibodies and acquired on a flow cytometer. The large majority of the Tregs were $CD62L^+$ (84.8% ± 2.2%) and $CCR4^+$

(78.5% ± 11.4%) (**Figures 6.1-A, C and D**) while a lower percentage of Teffs were CD62L^{+} (56.7% ± 12.6%) and CCR4^{+} (62.7% ± 1.6%) (**Figures 6.1-B- D**). To test whether Treg EVs also expressed CCR4, Treg EVs were attached to latex beads and immunostained and acquired on a flow cytometer. Treg EVs displayed a low expression level of CCR4 on their surfaces (**Figure 6.1-E**), suggesting that they have the potential bind to its ligands; CCL17 and CCL22, which are present in human skin (K. Fukuda et al. 2003; Tapia et al. 2007). Due to time constraints on this project the presence of CCR4 on Teff EVs and the expression of CD62L were not assessed on Treg EVs or Teff EVs.

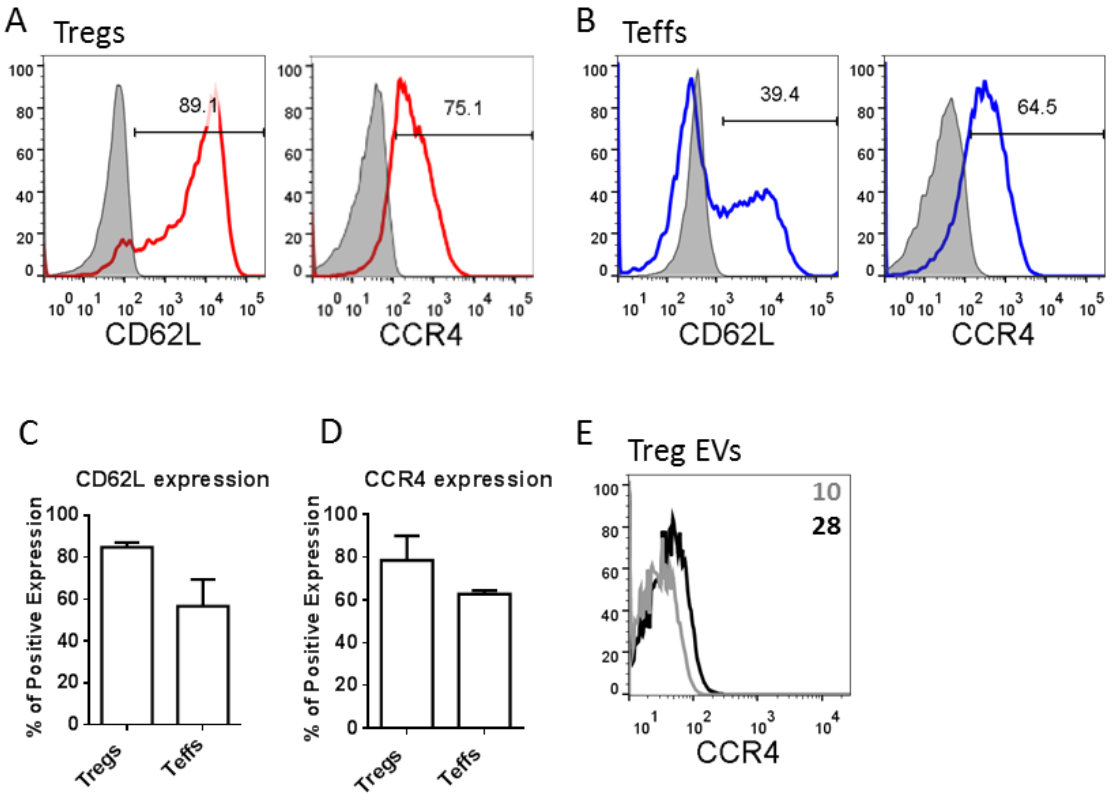


Figure 6.1- Human Tregs, Teffs and Treg EVs express homing receptors

***(A)** Human Tregs and **(B)** Teffs were expanded in vitro and at the end of culture were immunostained to test for the expression of CD62L and CCR4. The y-axis indicates the percentage of maximum expression. **(C and D)** The pooled data of percentage of cells that had positive expression levels on Tregs or Teffs for **(C)** CD62L and **(D)** CCR4. n= 3-4 per group. **(E)** Human Tregs were activated using plate-bound anti-CD3/CD28 antibodies and after 24 hours, the supernatant was collected and EVs purified using ultracentrifugation. EVs were attached to latex beads and immunostained for the*

presence of CCR4 expression. The y-axis indicates the percentage of maximum expression. Representative FACS plot from 4 independent experiments.

6.3.2 Human Treg EVs and Teff EVs function *in vivo* to prevent human skin allograft morphological damage

To investigate whether human Treg EVs elicited a similar protective role *in vivo* as observed *in vitro* in section 4.3.4 and section 4.3.7, we utilised a well-established humanised mouse model of skin transplantation (P. Sagoo et al. 2011; Putnam et al. 2013). In this model, recipient-derived T cells are the major driver of human skin transplant rejection.

Using this model, two preparations of Treg EVs were used due to previous reports describing the differences of EVs functionality depending on the EV isolation method used (Y.-T. Tang et al. 2017; Rekker et al. 2014). Therefore, it was important to determine whether the *in vivo* function of Treg EVs isolated by ExoQuick-TC™ (EQ) were similar to Treg EVs isolated by ultracentrifuge (ultra).

To test whether anything pelleted from the media using ExoQuick-TC™ was responsible for causing the response observed *in vivo*, the pellet resulting from the media alone was injected into the mice. This was arranged by preparing a pellet of media alone (no cells or EVs) mixed with ExoQuick-TC™. Using the above mentioned humanised mouse model of skin transplantation approach, human skin grafts were transplanted onto BRG immunodeficient mice and after 35 days, $5x10^6$ allogeneic human $CD25^-$ peripheral blood mononuclear cells (PBMCs) were adoptively transferred alone or in combination with $1x10^6$ autologous Tregs, $50x10^6$ Tregs-derived EVs isolated by ExoQuick-TC™ (EQ), $50x10^6$ Tregs-derived EVs isolated by ultracentrifugation (ultra), $50x10^6$ Teffs-derived EVs (EQ) or the equivalent amount of media alone used to isolate the EVs (media pellet (EQ)) (Figure 6.2).

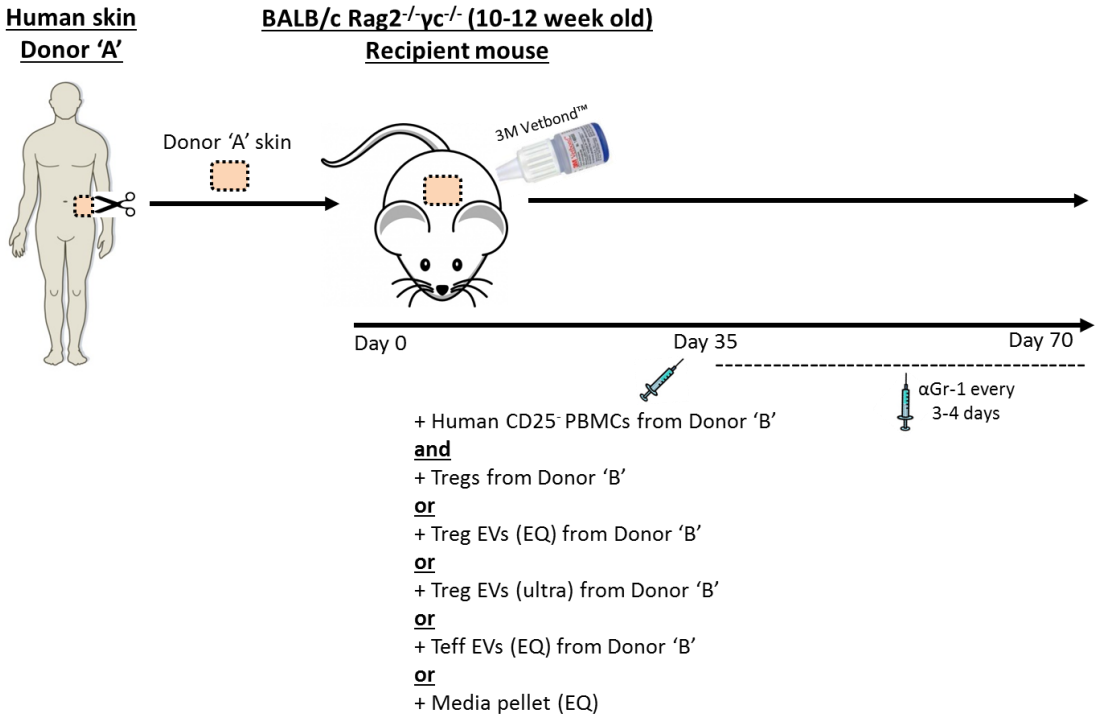


Figure 6.2- Humanised mouse model of skin transplantation

Diagram outlining the experimental set up and time-line of the human skin xenograft transplant and adoptive transfer of cells and EVs. BRG immunodeficient mice were transplanted with a section of human skin which was derived from healthy donors with written consent. After 35 days, the mice received allogeneic CD25- PBMCs, in the absence or presence of autologous 1x10^6 Tregs, 50x10^6 Tregs-derived EVs isolated by ExoQuick-TC™ (EQ), 50x10^6 Tregs-derived EVs isolated by ultracentrifuge (ultra), 50x10^6 Teffs-derived EVs isolated by EQ or the pellet resulting from media alone mixed with EQ (media pellet EQ). Cells and EVs were injected via the tail vein intravenous route. From that point onwards until day 70 post-transplant, the mice received 100µg of anti-mouse anti-Gr1 injection twice weekly via the intraperitoneal route.

As explained in section 6.1.1, the innate mouse cells are functional in these mouse models therefore to prevent the mouse cells from damaging the injected human cells; the transplanted mice received twice weekly injections of anti-mouse anti-Gr1 antibody from the time of injection of the human cells (day 30) until the end point of study; day 70 post-transplantation. Throughout the study, the mice did not exhibit symptomatic signs of GvHD and maintained stable body weight (Figure 6.3). Mice were culled 35 days

after the injection of the human cells and the skin allografts were assessed macroscopically for visible signs of skin damage (Appendix 6-A). The mice treated with PBMCs alone appeared to display visible signs of inflammatory damage compared to saline controls, however with the additional treatment of Tregs, Treg EVs (EQ), Treg EVs (ultra) and Teffs EVs (EQ), the visual appearance of inflammatory damage was reduced (Appendix 6-A). The parameters used to assess visual macroscopic inflammatory damage were the changes in skin colour; whereby skin that had darkened indicated higher levels of damage due to vessel ruptures caused by alloimmune mediated responses.

The ventral side of the human skin allograft were checked for visible vascular networks providing the allograft with blood supply which otherwise the allograft may deteriorate due to lack of bloody supply which could subsequently be mistaken for transplant rejection (Appendix 6-B).

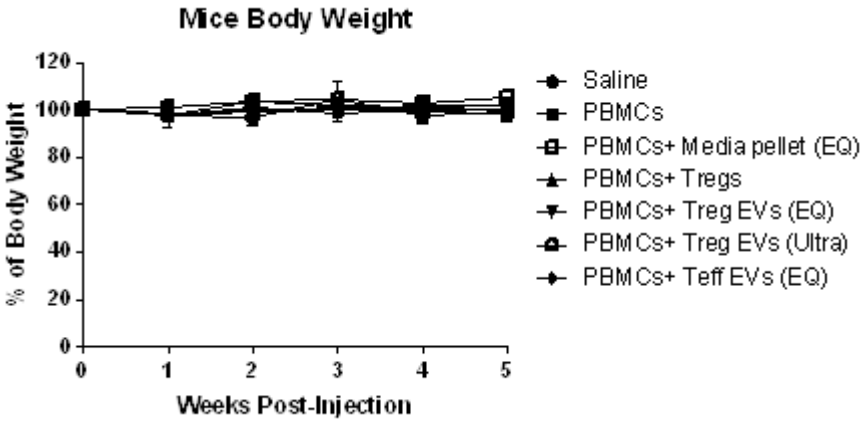


Figure 6.3- Mice body weight throughout study

The body weight of each mouse was recorded throughout the study. The initial weight of each mouse was recorded as 100%, and every week their weight was taken and presented as relative to the initial 100% weight. Bars show mean+ SEM. n= 3-9 per group.

Human skin allografts were analysed using various histological immunostaining parameters. To assess general morphological changes to the skin structures caused by

the various treatments, haematoxylin & eosin (H&E) staining was performed on cross-sections of the grafted human skin. The PBMCs-treated mice displayed an irregular stratum corneum, disrupted epidermis and dense cellular infiltration compared to saline-treated control mice (Figures 6.4-B and A, respectively). Additional treatment with Tregs, Treg EVs (EQ), Treg EVs (ultra) and Teff EVs (EQ) resulted in a uniform stratum corneum and the epidermis appeared regular with less cellular infiltration compared with PBMCs treated mice (Figures 6.4-D-G, respectively). Having confirmed that the EVs isolated from media alone did not contribute to inhibition of T cell proliferation *in vitro* (Figure 4.5-I), I next tested whether the media alone pellet affected the outcome *in vivo.* Mice treated with Media pellet (EQ) showed thickening of the epidermis layer akin to the PBMCs-treated controls (Figures 6.4-C and B, respectively) suggesting that the media pellet does not contribute to morphological changes seen with the EV preparations.

Morphometric measurements and statistical analysis of the epidermis architecture was performed using two assessment criteria; the epidermal thickness (without the rete ridges) and the height of the rete ridges from the base (Figure 6.4-H), similar to the histological assessment criteria employed by other groups (Giangreco et al. 2010; Chanadanwale et al. 2015).

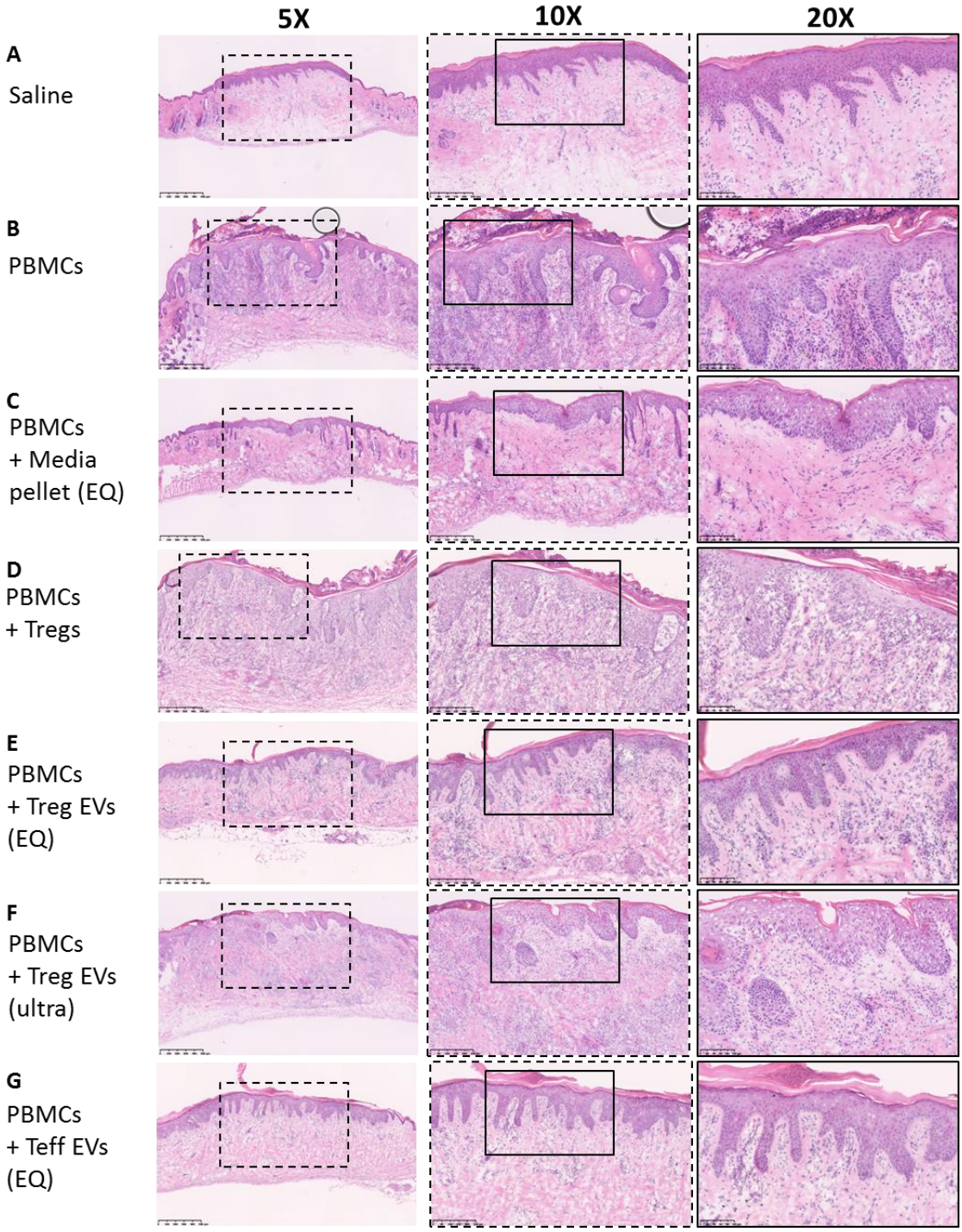
The epidermal thickness was significantly increased by the treatment of PBMCs alone or PBMCs+ Media pellet (EQ) as compared to saline controls ($p<0.0001$ and $p=0.0197$, respectively; Figure 6.4-I), indicating an alloimmune mediated inflammatory response. When PBMCs were co-injected with the addition of Tregs, Treg EVs (EQ), Treg EVs (ultra) or Teff EVs (EQ) this significantly decreased the epidermal thickness (all $p<0.0001$; Figure 6.4-I). When PBMCs+ Media pellet (EQ) was injected or PBMCs with Treg EVs (EQ) or Treg EVs (ultra), a significantly decreased epidermal thickness was observed ($p=0.0414$ and $p=0.0437$, respectively; Figure 6.4-I), indicating that Treg EVs function *in vivo*. However, the comparison PBMCs+ Media pellet (EQ) with PBMCs+ Teff EVs (EQ) resulted in no statistically significant difference (p=ns; Figure 6.4-I).

The rete ridges elongate from the epidermal to dermal regions to enhance the surface area of the capillary-epidermal junction, but irregular elongation of rete ridges are signs of inflammatory skin lesions (Lawlor and Kaur 2015). To test whether rete ridge heights were altered by the various treatments, the rete ridge heights were measured. The rete

ridge height was significantly increased by the treatment of PBMCs alone or PBMCs+ Media pellet (EQ) compared to saline (both p<0.0001; Figure 6.4-J), providing another indication of an alloimmune mediated inflammatory response. When PBMCs were co-injected with the addition of Tregs, Treg EVs (EQ), Treg EVs (ultra) or Teff EVs (EQ), the rete ridge heights were significantly decreased (all p<0.0001; Figure 6.4-J).

When PBMCs+ Media pellet (EQ) was injected or PBMCs with Treg EVs (EQ) or Treg EVs (ultra), a significantly decreased rete ridge height was also noted (p=0.0291 and p=0.0037, respectively; Figure 6.4-J), providing yet another indication that Treg EVs function *in vivo*. However, the comparison PBMCs+ Media pellet (EQ) with PBMCs+ Teff EVs (EQ) resulted in no statistically significant difference (Figure 6.4-J).

Overall, the data suggests that human Treg EVs and Teff EVs may protect against alloimmune mediated human skin allograft damage as assessed macroscopically and morphologically.


5X
10X
20X
A
Saline
B
PBMCs
C
PBMCs
+ Media
pellet (EQ)
D
PBMCs
+ Tregs
E
PBMCs
+ Treg EVs
(EQ)
F
PBMCs
+ Treg EVs
(ultra)
G
PBMCs
+ Teff EVs
(EQ)

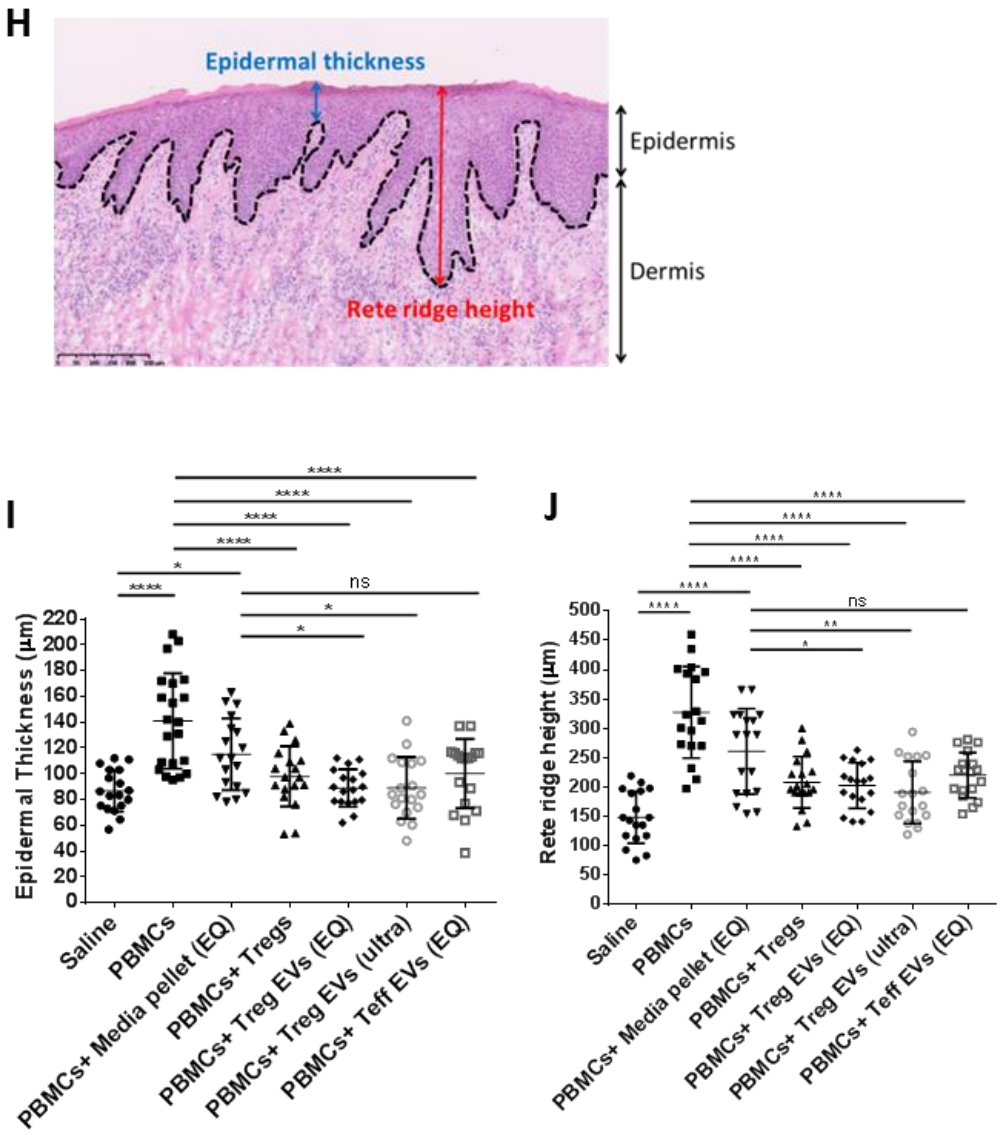


Figure 6.4- Human Treg EVs prevented alloimmune mediated morphological damage of human skin

Transplanted human skin allografts were harvested 35 days post- cells/EVs injection and stained with haematoxylin and eosin (H&E) and acquired using a high definition scanning light microscope. Representative images are shown. The left panel indicates a 5X objective with a scale bar of 500µm, the dotted box is enlarged and displayed in the middle panel which indicates a 10X objective with a scale bar of 250µm. The lined box is enlarged and displayed in the right panel which indicates a 20X objective with a scale bar of 100µm. The H&E images show mice treated with ***(A)*** *Saline* ***(B)*** *PBMCs* ***(C)*** *PBMCs+ Media pellet (EQ)* ***(D)*** *PBMCs+ Tregs* ***(E)*** *PBMCs+ Treg EVs (EQ)* ***(F)*** *PBMCs+ Treg EV (ultra) and* ***(G)*** *PBMCs+ Teff EVs (EQ).* ***(H)*** *An example of how the morphological and*

*histological assessment of epidermis was performed. The blue arrow indicates the start and end point for measuring epidermal thickness and the red arrow indicates the start and end point for measuring rete ridge height. Graphs show **(I)** the epidermal thickness and **(J)** rete ridge height of the various treated mice. Bars show mean ± SEM. For each condition, the number of mice used were; Saline n=7, PBMCs n=9, PBMCs+ Media pellet n=3, PBMCs+ Tregs n=8, PBMCs+ Treg EVs (EQ) n=5, PBMCs+ Teff EVs (EQ) n=4 and PBMCs+ Treg EVs (Ultra) n=3. Statistical significance was tested using one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= non-significant.*

6.3.3 Human Treg EVs and Teff EVs protect against alloimmune mediated human skin allograft damage by reducing immune cell infiltration

To further investigate how human Treg EVs inhibited alloimmune mediated human skin allograft damage I used a panel of immunofluorescence staining parameters to test various markers previously used to assess skin graft condition (P. Sagoo et al. 2011; Putnam et al. 2013). These staining panels included: CD45, a common leukocyte antigen marker; CD3, a T cell marker; Ki67, a cell proliferation marker (proliferating keratinocytes); CD31, an endothelial marker indicating the integrity of blood vessels and involucrin, a human skin protein marker present in the epidermis.

A significant increase of $CD45^+$ immune cells and $CD3^+$ T cells was observed in the PBMCs-treated mice compared to saline-treated control mice ($CD45^+$ cells $p<0.0001$ and $CD3^+$ cells $p<0.0001$; **Figures 6.5-A, B, H and I**). In addition, the $Ki67^+$ proliferating keratinocytes were also significantly increased in the PBMCs-treated mice compared to saline-treated control mice ($p<0.0001$; **Figures 6.5-A, B and J**). Together, this demonstrated that the transfer of allogeneic PBMCs induced a high level of infiltration of cells, linking to the inflammatory damage observed macroscopically and morphologically (**Appendix 6-A and Figures 6.4-A and B**).

The mice which received PBMCs with the addition of Tregs, Treg EVs (EQ), Treg EVs (ultra) or Teff EVs (EQ) had significantly decreased numbers of infiltrating $CD45^+$ cells compared to PBMCs alone (Tregs $p=0.0026$; Treg EVs (EQ) $p<0.0001$; Treg EVs (ultra) $p=0.0016$; Teff EVs (EQ) $p=0.0003$; **Figure 6.5-H**), which correlated with significantly fewer infiltrating $CD3^+$ T cells (Tregs $p=0.0058$; Treg EVs (EQ) $p<0.0001$; Treg EVs (ultra)

p=0.0001; Teff EVs (EQ) p<0.0001; Figure 6.5-I). Notably, when Tregs were adoptively transferred into the hosts, the reduction of infiltrating $CD45^+$ and $CD3^+$ cells were not as pronounced as expected (P. Sagoo et al. 2011; Putnam et al. 2013; Boardman et al. 2017) as the adoptive transfer of Treg EVs (EQ) (For $CD45^+$; Tregs p=0.0026 and Treg EVs (EQ) p<0.0001; For $CD3^+$; Tregs p=0.0058 and Treg EVs (EQ) p<0.0001), which may be due to the Tregs inducing a population of adaptive Tregs within the transplanted allograft, as previously demonstrated by the host laboratory (P. Sagoo et al. 2011; Putnam et al. 2013; Boardman et al. 2017).

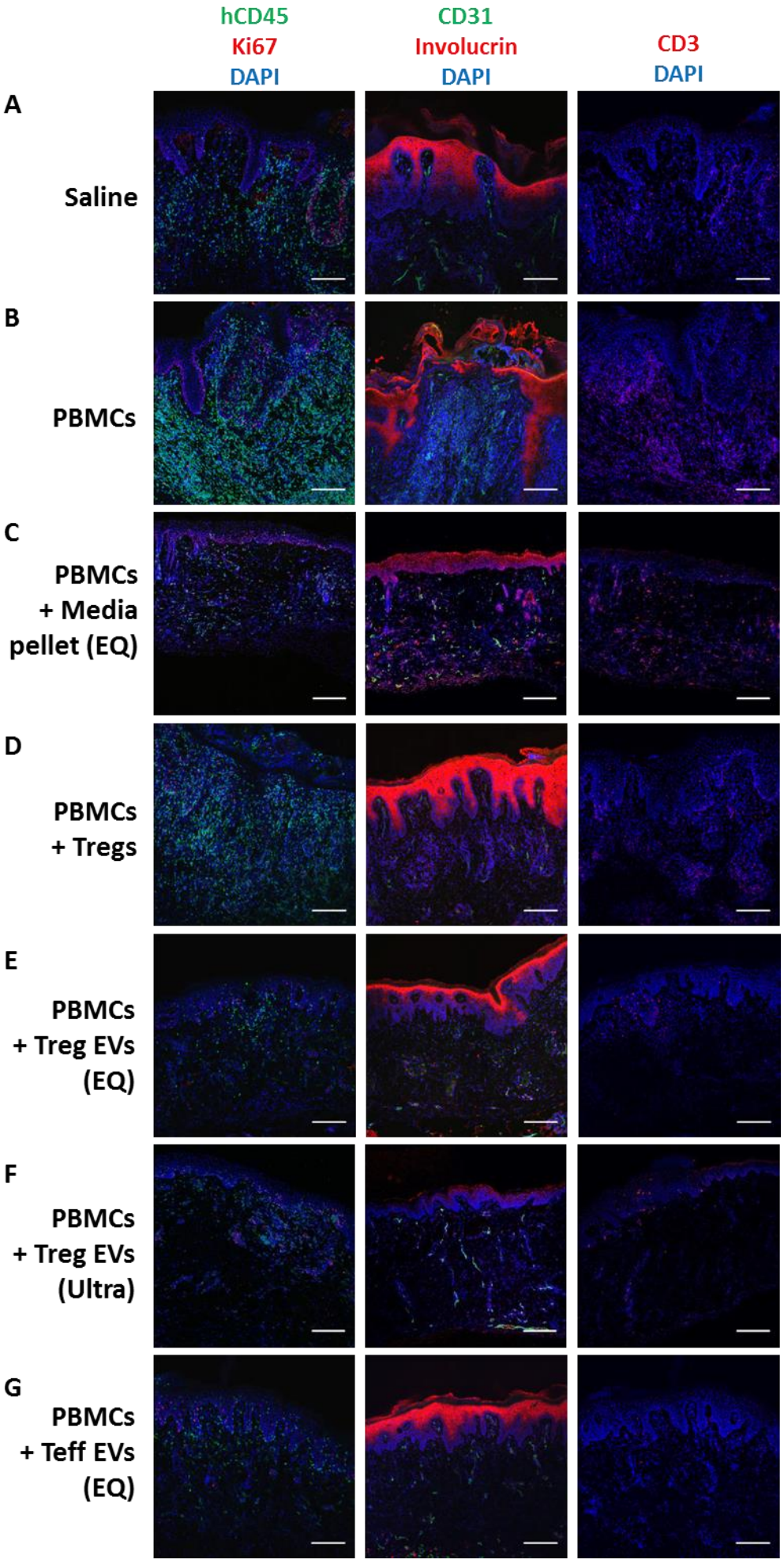
Furthermore, I also observed a significantly decreased frequency of $Ki67^+$ proliferating keratinocytes with the addition of Tregs, Treg EVs (EQ), Treg EVs (ultra) or Teff EVs (EQ) compared to PBMCs alone (Tregs p<0.0001; Treg EVs (EQ) p<0.0001; Treg EVs (ultra) p=0.0006; Teff EVs (EQ) p=0.0001; Figure 6.5-J). The involucrin-expressing epidermal layer was intact in the saline treated mice but was partially lost or defected in PBMCs treated mice, indicating signs of damage. This level of damage (as observed by involucrin) was not observed in the presence of Tregs, Treg EVs (EQ), Treg EVs (ultra) or Teff EVs (EQ) (Figures 6.5-B, D-G). The CD31 endothelial marker indicating the integrity of blood vessels in the saline treated mice were intact and had a uniform vascular structure but this was clustered in the PBMCs treated mice, indicating alloimmune damage. These vessels were protected from damage by the presence of Tregs, Treg EVs (EQ), Treg EVs (ultra) or Teff EVs (EQ) (Figures 6.5-B, D-G).

Additional treatment with the Media pellet (EQ) had no significant effect on the infiltration of cells compared to PBMCs alone as measured by the frequency of $CD45^+$ cells, $Ki67^+$ cells and $CD3^+$ cells (Figures 6.5-H-J). Hence the background control of Media pellet (EQ) does not contribute to the effects observed by Treg EVs (EQ) nor Teff EVs (EQ). However, when statistical testing was performed for $CD45^+$ frequencies for PBMCs+ Media pellet (EQ) and PBMCs+ Treg EVs (EQ) or PBMCs+ Teff EVs (EQ) it resulted in p=0.0036 and p=ns, respectively (Figure 6.5-H). Similarly, for $CD3^+$ frequencies, PBMCs+ Media pellet (EQ) and PBMCs+ Treg EVs (EQ) or PBMCs+ Teff EVs (EQ) it resulted in p=0.0016 and p=0.0172, respectively (Figure 6.5-I). Whereas, for $Ki67^+$ frequencies, PBMCs+ Media pellet (EQ) and PBMCs+ Treg EVs (EQ) or PBMCs+ Teff EVs (EQ) it resulted in p<0.0001 and p=0.0027, respectively (Figure 6.5-J). Taken together, when comparing these p-values with those obtained by comparing PBMCs

alone with each of the treatment groups, the p-values from PBMCs alone were more statistically significant compared to the p-values obtained when comparing PBMCs+ Media pellet (EQ) with each treatment group, which might indeed be due to some minimal effects from the Media pellet (EQ).

Nonetheless, there was no significant difference between Treg EVs (EQ) and Treg EVs (ultra) when injected *in vivo* and assessed for the frequency of $CD45^+$ cells, $Ki67^+$ cells and $CD3^+$ cells (Figures 6.5-H-J). Furthermore, there was no significant difference between Treg EVs (EQ) and Teff EVs (EQ) treatment when assessed for the frequency of $CD45^+$ cells, $Ki67^+$ cells and $CD3^+$ cells (Figures 6.5-H-J), thus Treg EVs (EQ) and Teff EVs (EQ) have similar effects *in vivo*.

Overall, the adoptive transfer of human Treg EVs, isolated by ExoQuick-TC™ or ultracentrifuge, and Teff EVs protected against human skin damage mediated by alloreactive $CD3^+$ T cells. Human Treg EVs reduced the numbers of $CD45^+$ immune cells, $CD3^+$ immune cells and $Ki67^+$ keratinocytes within the allograft, suggesting that human Treg EVs may have a role in promoting transplant tolerance by inhibiting intra-graft cell infiltration.


hCD45
Ki67
DAPI
CD31
Involucrin
DAPI
CD3
DAPI
A
Saline
B
PBMCs
C
PBMCs
+ Media
pellet (EQ)
D
PBMCs
+ Tregs
E
PBMCs
+ Treg EVs
(EQ)
F
PBMCs
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PBMCs
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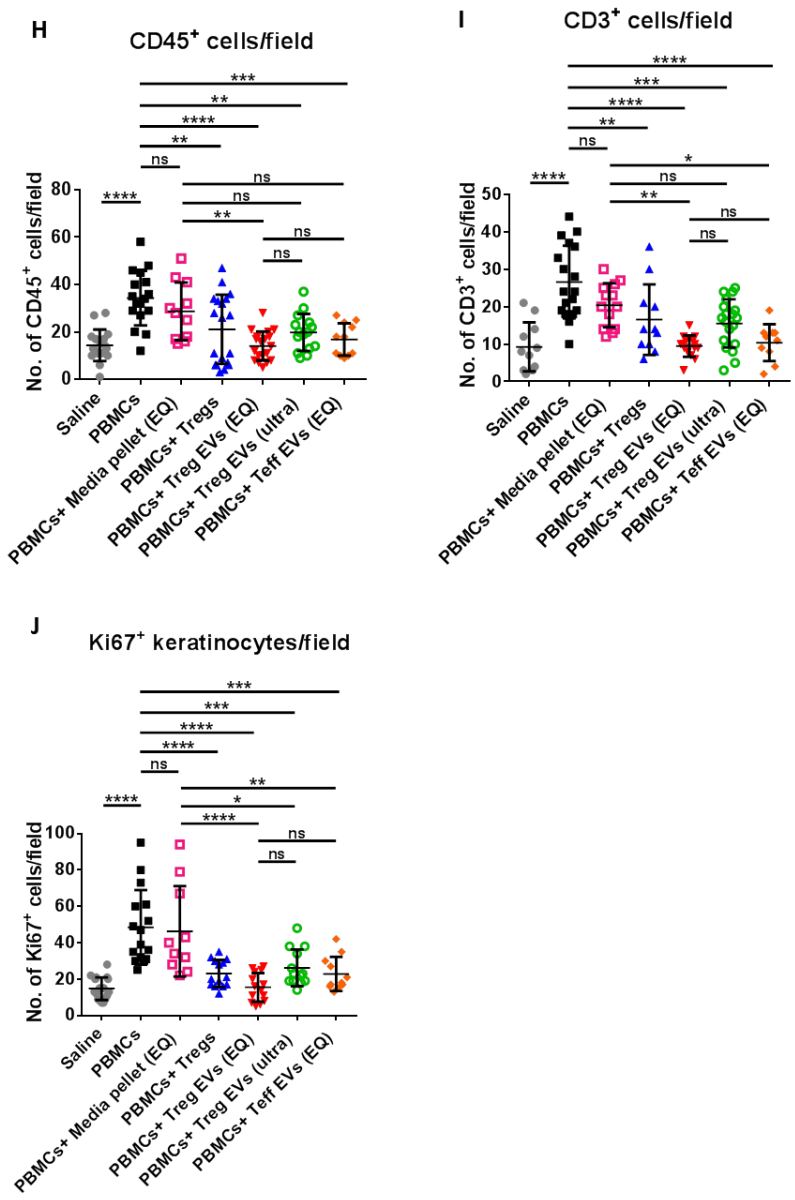

Figure 6.5- Human Treg EVs protect against alloimmune mediated human skin allograft damage by reducing the infiltration of immune cells and inhibiting the proliferation of keratinocytes

(A-G) *Representative confocal immunofluorescence images of human skin graft sections fixed and stained for (first column) human CD45 (green), Ki67 (red) and DAPI (blue); (second column) CD31 (green), involucrin (red) and DAPI (blue); (third column) human CD3 (red) and DAPI (blue) with the various indicated treatments. Mice were treated with* ***(A)*** *saline* ***(B)*** *PBMCs* ***(C)*** *PBMCs+ Media pellet (EQ)* ***(D)*** *PBMCs+ Tregs* ***(E)*** *PBMCs+ Treg EVs (EQ)* ***(F)*** *PBMCs+ Treg EVs (ultra) and* ***(G)*** *PBMCs+ Teff EVs (EQ). Quantification of the numbers of* ***(H)*** *human CD45⁺ cells* ***(I)*** *CD3⁺ cells and* ***(J)*** *Ki67⁺ cells per field of view was performed using NIS Elements and FIJI imaging software. Results represent 3-9 mice*

*per group where two to three fields of view were quantified per section and data are representative of 5 individual experiments. For each condition, the number of mice used were; Saline n=7, PBMCs n=9, PBMCs+ Media pellet n=3, PBMCs+ Tregs n=8, PBMCs+ Treg EVs (EQ) n=5, PBMCs+ Teff EVs (EQ) n=4 and PBMCs+ Treg EVs (Ultra) n=3. Statistical significance was tested using one-way ANOVA where *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 and ns= non-significant.*

6.3.4 Human Tregs can be labelled with eGFP-CD63 and mCherry-CD63

Given the above findings it could be envisaged that Treg EVs may have migrated to the site of transplanted tissue to exert their suppressive function. Thus, to determine whether Treg EVs migrated to the transplanted human skin tissue or whether these migrated to secondary lymphoid organs such as the spleen or lymph nodes, Treg EVs were labelled with eGFP-CD63 or mCherry-CD63 to initially assess which construct provided the best transduction efficiency. To this end, Tregs were transduced with eGFP-CD63 or mCherry-CD63 lentiviral particles with the aim to track their migration and uptake by target T cells. Given that Tregs and Teffs are difficult to distinguish, I aimed to transduce Tregs with eGFP-CD63 (or mCherry-CD63 if this provided a higher transduction efficiency) and T cells with the other construct to discriminate the two EV populations and to visualise the transfer of EVs (Figure 6.6). Furthermore, the process of Treg release of EVs and their uptake by target T cells has not previously been imaged. As shown in Figures 4.2 and 4.3, human Tregs and Teffs express CD63 both on the extracellular and intracellular levels.

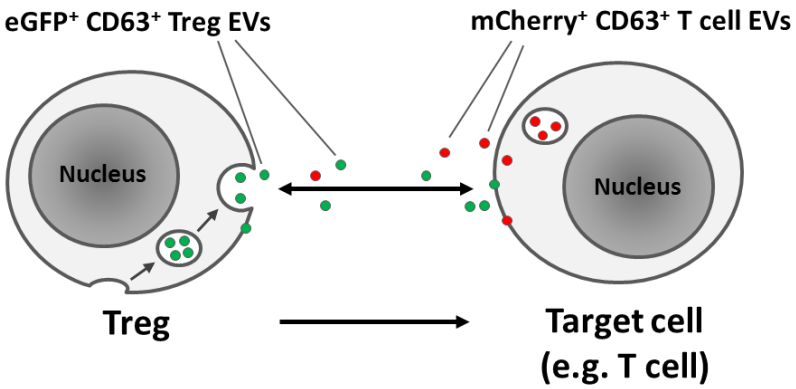

Figure 6.6- Diagram of labelled Treg EVs

Diagram showing the aim to visualise the interaction of pre-labelled Treg EVs (eGFP-CD63$^+$) and target cells (e.g. T cells). To discriminate between the different EVs (in case of EV cross-talk between the two cells), target cells will be labelled with mCherry-CD63$^+$ to obtain mCherry-CD63$^+$ EVs.

The eGFP-CD63 and mCherry-CD63 vector constructs were a kind gift from Professor Tony Ng, King's College London. A vector map of eGFP-CD63 and mCherry-CD63 are included in Appendix 1 and 2, respectively. To prepare plasmid DNA, maxipreps were processed and restriction analysis using EcoRI-HF® and AgeI-HF® enzymes performed on the selected clones to confirm the correct DNA was purified (Appendix 7). HEK293Ts are highly transfectable cells derived from HEK293 cells (Russell et al. 1977) which have been modified to stably express the Simian Vacoulating Virus 40 (SV40) large T-antigen. The presence of the SV40 T-antigen allows this cell line to replicate vectors carrying the SV40 region of replication (DuBridge et al. 1987; Pear et al. 1993) and able to support high titre lentivirus and retrovirus production. Thus, HEK293Ts were used as the viral particle producer cell line due to their efficiency in high titre viral particle production. HEK293Ts were transfected with a 3rd generation lentiviral system using 4 plasmids. These 4 plasmids consisted of pVSV-g (envelope vector), pRRE and pREV (packaging vectors) (a kind gift from Dr Gilbert Fruhwirth, King's College London) and eGFP-CD63 or mCherry-CD63 (gene of interest vectors). 48 hours post transfection, HEK293Ts expressed either eGFP or mCherry, demonstrating that they were successfully transfected with these plasmids (Figures 6.7-A and B, respectively). After confirmation

of eGFP and mCherry expression, the supernatant containing eGFP-CD63^{+} or mCherry-CD63^{+} viral particles were harvested and concentrated.

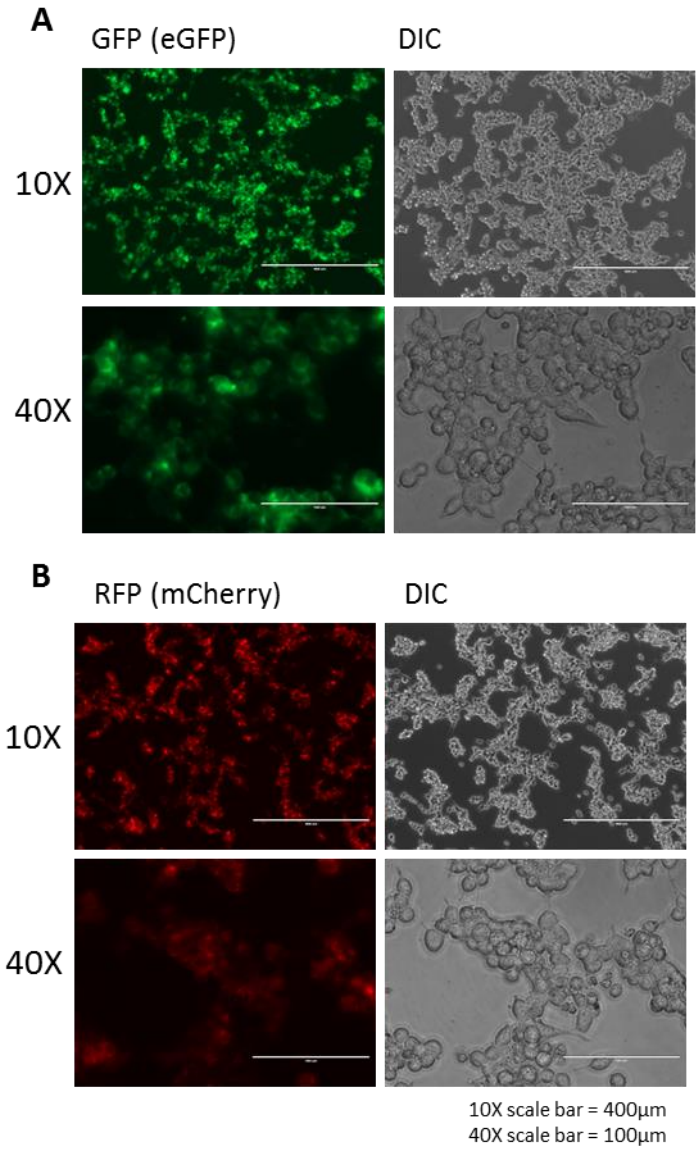

Figure 6.7- Transfection of HEK293Ts with eGFP-CD63 and mCherry-CD63

HEK293Ts were transfected with pVSV-g (envelope vector), pRRE and pREV (packaging vectors) and ***(A)*** *eGFP-CD63 or* ***(B)*** *mCherry-CD63 vectors. Top panels show 10X objectives with a scale bar of 400µm and bottom panels show 40X objectives with a scale bar of 100µm. Left panels show the respective fluorescence detected using GFP or RFP filters and right panels show the corresponding differential interference contrast (DIC) images.*

Three days post human Treg isolation; Tregs were transduced with various volumes of viral particles at 30µl, 50µl and 80µl to test the effect of increasing viral particle titre and the corresponding transduction efficiency. Seven days later, the transduction efficiency was assessed by flow cytometry. The transduction efficiency correlated with the volumes of eGFP-CD63 viral particles, namely 30µl of viral particles achieved 41.4% transduction efficiency, whereas 50µl achieved 46.8% and 80µl achieved 55.3% (Figure 6.8-A). The positive expression of eGFP was further confirmed by fluorescence microscopy (Figure 6.8-B). Similarly, the trend of higher viral particle titre with higher transduction efficiency was also observed for Tregs transduced with mCherry-CD63 viral particles (30µl of viral particles achieved 21.1%, whereas 50µl achieved 26.7% and 80µl achieved 30.3%) (Figure 6.8-C). This result was also confirmed by fluorescence microscopy (Figure 6.8-D). Ten days post-transduction, the cells were assessed by flow cytometry to check the percentage of cells positive for eGFP and mCherry expression (Figures 6.9-A and B, respectively). As observed, the percentage of cells positive for eGFP and mCherry had notably decreased from day seven to day ten post-transduction (Figures 6.8 and 6.9), possibly due to the untransduced cells having a survival, growth or expansion advantage over transduced cells as cells that have been transduced may slow their metabolism or general cell functions. To expand a pure population of eGFP-CD63^{+} and mCherry-CD63^{+} Tregs, transduced cells were FACS-sorted on day eleven, post transduction, based on their positive and high expression of eGFP and mCherry.

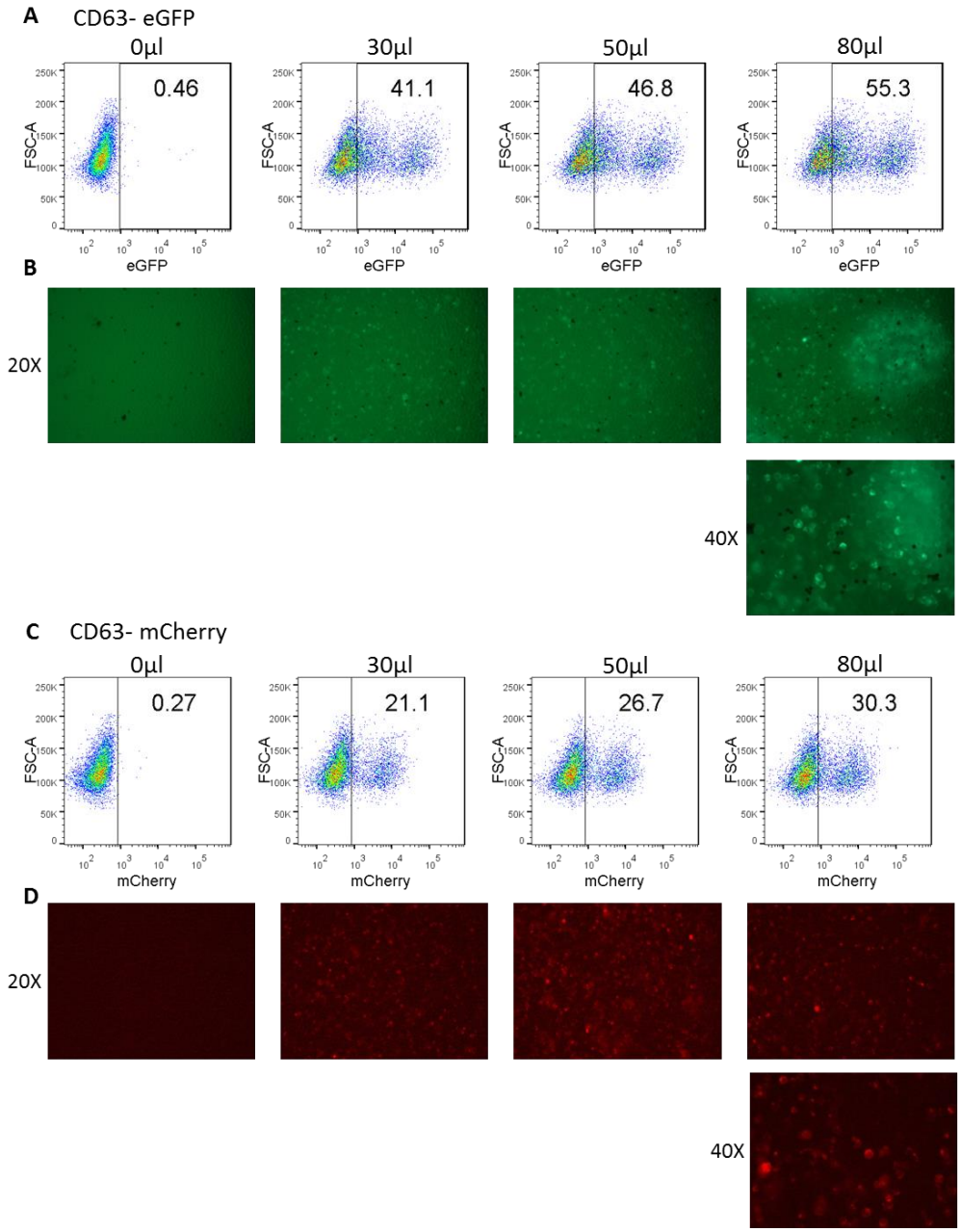

Figure 6.8- Transduction efficiency of human Tregs with CD63-eGFP and CD63-mCherry viral particles 7 days post-transduction

*Human Tregs were transduced with various volumes of viral particles of 0µl, 30µl, 50µl or 80µl of eGFP-CD63 or mCherry-CD63 and 7 days post-transduction, the transduction efficiency was assessed by gating on eGFP or mCherry expressing cells and the values of transduction efficiency are indicated. eGFP-CD63 was assessed by **(A)** flow cytometry and **(B)** fluorescence microscopy. mCherry-CD63 was assessed by **(C)** flow cytometry and **(D)** fluorescence microscopy.*

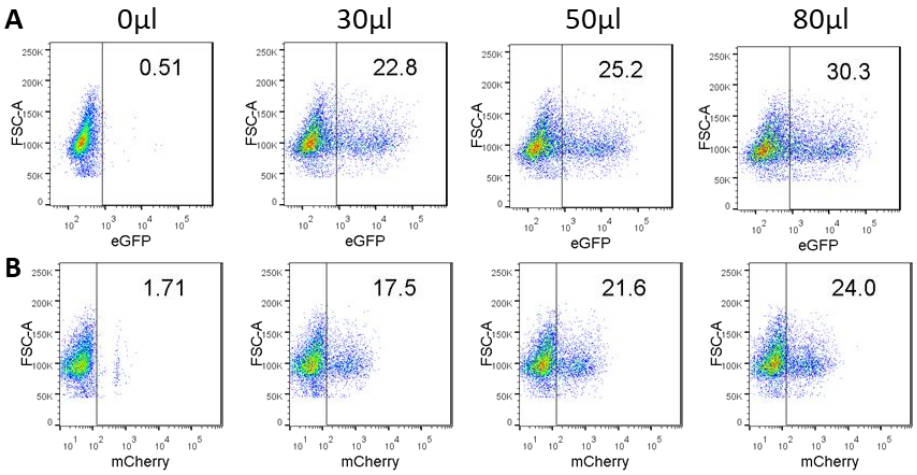

Figure 6.9- Transduction efficiency of human Tregs with CD63-eGFP and CD63-mCherry viral particles 10 days post transduction

*Various volumes of viral particles of 0µl, 30µl, 50µl or 80µl of eGFP-CD63 or mCherry-CD63 were used to transduce human Tregs. Human Tregs were assessed for transduction efficiency before FACS-sorting, 10 days post transduction by gating on eGFP or mCherry expressing cells and the values of transduction efficiency are indicated for **(A)** eGFP-CD63 and **(B)** mCherry-CD63.*

The eGFP$^+$ and mCherry$^+$ Tregs showed >98% purity (Figure 6.10). Tregs were expanded further using anti-CD3/CD28 beads, rapamycin and IL-2 for an additional 10 days, as described in section 2.13. At the end of culture, 21 days post-transduction, Tregs were assessed for their eGFP and mCherry positive expression before Tregs were activated to release EVs. Figure 6.11 shows that the percentage of eGFP and mCherry positive cells had reduced from >98% to 58.2% and 31.4%, respectively. Nonetheless, Tregs were activated and isolated EVs were attached to latex beads and assessed for endogenous CD63 eGFP$^+$ or CD63 mCherry$^+$ expression or were stained with anti-CD63 antibodies in the case of EVs derived from untransduced Tregs. As expected EVs derived from untransduced Tregs were CD63$^+$, but EVs derived from eGFP$^+$ or from mCherry$^+$ Tregs were neither eGFP nor mCherry positive (Figure 6.12-A). To confirm that the EVs were successfully adhered to the latex beads, the EVs were immunostained for the presence of CD81. The EVs derived from untransduced Tregs were CD81$^+$ but the CD81 expression on EVs derived from eGFP$^+$ Tregs or mCherry$^+$ Tregs was minimal (Figure 6.12-B). These findings suggest that CD63 transduced Tregs may have had a defect in EV

release or alternatively only EVs derived from transduced Tregs had adhered to the latex beads with all labelled EVs not efficiently attached. However, this experiment was derived from 1 donor, and therefore to draw conclusions, more donors and repeat experiments would be required.

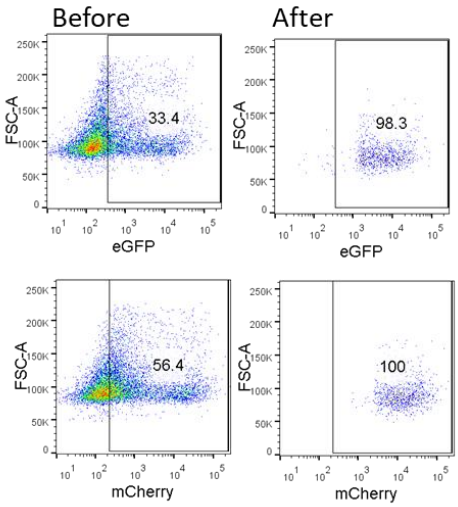

Figure 6.10- eGFP and mCherry expression levels in Tregs before and after FACS-sorting

Transduced human Tregs were assessed for transduction efficiency by eGFP or mCherry expression levels before and after FACS-sorting.

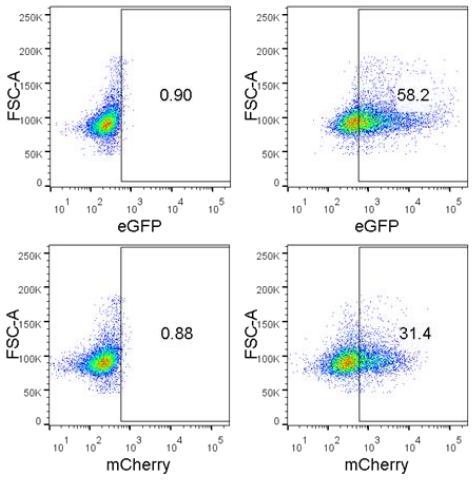

Figure 6.11- eGFP and mCherry expression levels in Tregs before cell activation for EV release

Transduced human Tregs were assessed for eGFP or mCherry expression levels before cell activation for EV release.

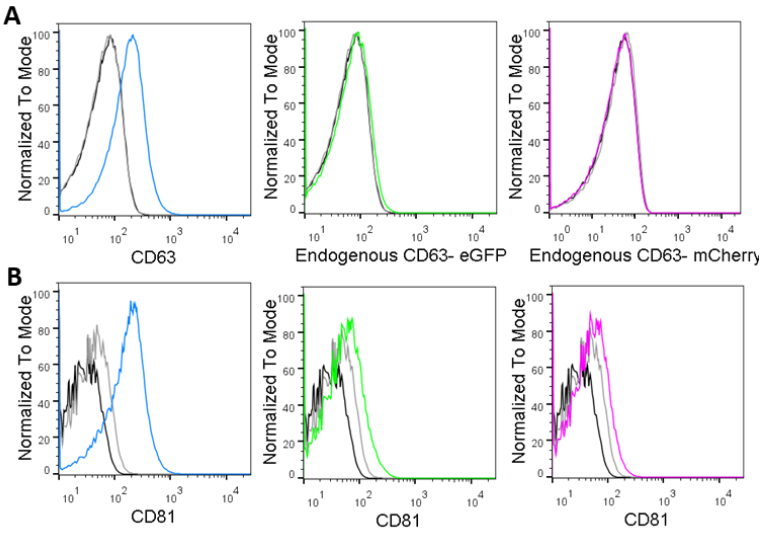

Figure 6.12- Transduced Treg EVs expression of endogenous CD63

Untransduced and transduced Tregs were activated with anti-CD3/CD28 antibodies and EVs were harvested and adhered to latex beads before immunostaining. ***(A)*** *Untransduced Treg EVs were tested for the presence of CD63 by antibody staining, whereas transduced Tregs were tested by the presence of endogenous eGFP or mCherry expression.* ***(B)*** *All EVs were tested for the presence of CD81 via antibody staining. Black lines indicate beads alone, grey line indicates beads stained with CD81 antibodies and coloured lines indicate EVs adhered to beads.*

6.4 Discussion

In this PhD thesis chapter, I focused on the question that has not been published so far, namely whether human Treg EVs function *in vivo* and whether human Treg EVs can suppress the pro-inflammatory microenvironment to promote allograft survival. In this chapter, I have demonstrated that human Treg EVs express the skin homing receptor CCR4. Importantly, I have shown that the adoptive transfer of human Treg EVs (but also Teff EVs) protected against alloimmune mediated human skin damage, indicating that human Treg EVs can suppress the pro-inflammatory microenvironment and thus increase the longevity of transplanted allografts. The Treg EVs and Teff EVs inhibited cellular infiltration within the transplanted human skin graft by preventing the presence and/or expansion of $CD45^+$ leukocytes, $CD3^+$ T cells and Ki67 proliferating keratinocytes. The results in this chapter extended what I presented in **Chapter 4- sections 4.3.4 and**

4.4.7, namely that human Treg EVs were immunosuppressive *in vitro*. It also confirmed that Teff EVs also function *in vivo*. Furthermore, I showed that human Tregs can be labelled with eGFP-CD63 and mCherry-CD63 lentiviral particles and could provide a tool for labelling their EVs, although more experimental optimisation was required.

The humanised mouse model used in this study only addressed the effect of the direct alloresponse. Although, as discussed in section 1.3, the direct allorecognition pathway is involved mainly in the acute rejection, whereas, in chronic rejection involves mainly the indirect allorecognition pathway. Further advancements in the humanised mouse models are required to address the function of EVs in regulating the indirect allorecognition pathway.

6.4.1 Homing receptors

For EVs to function efficiently, the EVs may have to migrate to the site of interest. For Tregs EVs this could be achieved by the expression of homing receptors, as has been shown in Tregs (Scottà et al. 2013). Homing receptors have previously been discovered in DC EVs by Wei *et al*. Wei *et al*. demonstrated that CCR7 expression on DC EVs allowed their migration into the spleen and induced inflammation at the site (G. Wei et al. 2017). T cells secrete exosomes containing CXCR4 (Blanchard et al. 2002), possibly to enhance T cell exosomes' ability to home to specific locations where chemokines are secreted. However, the specific destination of Treg EVs after adoptive transfer into mice is unknown. Although the expression of homing receptors has been described in T cell EVs (Guillaume van Niel, D'Angelo, and Raposo 2018), the literature so far to date has not unravelled the presence of homing receptors in Treg derived EVs. I found low expression levels of CCR4 on Treg EVs, given that the parent Tregs have high expression of this chemokine receptor that allows their homing to lymphoid organs and to skin it is a possibility that CCR4 facilitated their uptake into lymphoid organs whereby they can action their suppressive functions, although this was not tested. Importantly, when Treg EVs were adoptively transferred into the mice a level of protection within the allograft was mediated suggesting that Treg EVs exert their suppressive function towards the transplanted human skin.

6.4.2 Suppressive ability of Treg EVs

The microscopic analysis of the transplanted mice skins showed that the addition of Treg EVs (EQ) and Treg EVs (ultra) had protected against inflammatory damage compared to PBMCs alone control. As there was no difference between Treg EVs purified via ExoQuick-TC™ or ultracentrifuge *in vivo,* it could be concluded that these two EV isolation methods are comparable at least for *in vivo* assessments. Other studies have used ExoQuick-TC™ and ultracentrifuge isolated EVs to assess their different responses *in vitro* (Okoye et al. 2014), however, very few studies have used direct comparison of ExoQuick-TC™ versus ultracentrifuge in the *in vivo* setting.

Both Treg EVs and Teff EVs protected against alloreactivity *in vivo* by preventing the infiltration of $CD45^+$ and $CD3^+$ cells within the allograft. Furthermore, $Ki67^+$ proliferating keratinocytes were also significantly reduced in the presence of Treg EVs and Teff EVs, suggesting that Treg EVs and Teff EVs may exert their inhibitory effects in non-immune cells as well as immune cells. Given that in section 4.3.7, I showed that Treg EVs and Teff EVs functioned differently in terms of affecting the cytokine production by Tresps, in this chapter both Treg EVs and Teff EVs functioned similarly *in vivo*.

Given that other studies have shown that Teff EVs can be both pro-inflammatory or anti-inflammatory (see section 1.7.7), it suggests that parent Teffs are somewhat 'plastic' and thus can produce EVs that function according to their parent cell status and thus Teff EVs can promote or suppress the immune response. On the contrary, all 7 publications to date regarding mouse, rat and human Treg EVs have collectively demonstrated that Treg EVs are immunosuppressive and not pro-inflammatory (Tung et al. 2018; Lesley Ann Smyth et al. 2013; Okoye et al. 2014; X. Yu et al. 2013; Aiello et al. 2017; Torri et al. 2017; Azimi et al. 2018). Therefore, whether this favours Treg EVs over Teff EVs as an immunotherapy for promoting transplantation tolerance is plausible.

Despite very similar behaviour *in vivo*, human Treg EVs and Teff EVs have different mechanisms of action as the Teff EVs promoted rather than suppressed the production levels of IFNγ and IL-6 (see section 4.3.7). Furthermore, in Chapter 5, it was shown that Teff EVs contain different miRNA repertoires compared to Treg EVs. Furthermore, and as displayed in Figure 5.7, bioinformatics analysis predicted that miRNAs enriched in Treg EVs can possibly inhibit the expression of genes associated in allograft rejection

and T cell receptor signalling pathways. As listed in Table 5.5, Teff EVs also contained high expression levels of miR-1972 compared to parent Teffs; miR-1972 was *in silico* predicted to target genes associated with the allograft rejection pathway which may provide an explanation of how Teff EVs functioned *in vivo* to protect allograft inflammatory damage. Collectively, these findings could potentially link to the *in vivo* protection provided by Treg EVs and Teff EVs against alloimmune mediated allograft damage and rejection observed in this PhD thesis chapter. As listed in Table 5.4, miR-126 is enriched in Treg EVs, and Wang *et al*. demonstrated that miR-126 governs vascular integrity and angiogenesis (S. Wang et al. 2008). The presence of miR-126 in Treg EVs might explain how these EVs were able to inhibit allogeneic PBMCs from damaging $CD31^+$ vessels as observed.

Whether Treg EVs can replace Tregs as a therapy is uncertain but given that Tregs may be unstable and that Treg EVs function *in vivo* to prevent allograft inflammatory damage, it has the potential to be used as a combinatory therapy with Tregs to maintain their suppressive potency. Whether Treg EVs can be used as a standalone therapy is uncertain due to Tregs being more potent at suppressing Tresps proliferation but nonetheless, Treg EVs are immunosuppressive both *in vitro* and *in vivo*. This could potentially be thought of as a cell-free maintenance therapy whereby Treg EVs can be injected *in vivo* to maintain the longevity of an allograft.

6.4.3 Labelling of EVs

To further clarify the mechanisms of action of EVs and to track their migration and destination in transplanted mice, Treg EVs were labelled with eGFP-CD63 and mCherry-CD63 with the aim to apply whole body optical imaging of GFP^+ or RFP^+ EVs (M. Yang et al. 2000). Although as presented in Figure 6.12, EVs derived from the transduced Tregs did not display eGFP or mCherry positive expression. Whether this was due to technical reasons or the biology of the transduced Tregs is not clear as only 1 donor sample was used, further donors will be required to enhance the isolation of eGFP or mCherry positive EVs. Nonetheless, the data acquired suggested that Tregs can be successfully labelled with CD63-eGFP or CD63-mCherry viral particles and thus has potential to be tracked *in vivo* and warrants further investigation.

EVs derived from other cell types have been tracked *in vivo*. Takahashi *et al.* showed that following the intravenous injection of cancer exosomes, these vesicles migrated firstly towards the liver and then to the lungs (Y. Takahashi et al. 2013). Exosomes derived from MSC have also been imaged and these EVs migrated to the kidney and spleens of mice undergoing acute kidney injury (GRANGE et al. 2014). Whether Tregs and their EVs migrate to the same location is unknown. But others have demonstrated that directional cell movement, mobility, motility and chemotaxis, are enhanced by exosomes released by their parent cells; cancer cells, neutrophils and endothelial cells (Sung et al. 2015; Majumdar, Tavakoli Tameh, and Parent 2016; Sung and Weaver 2017; Brown et al. 2018)

Imaging of Tregs has been performed by the host laboratory by Sharif-Paghaleh *et al*. (Sharif-Paghaleh et al. 2011). Sharif-Paghaleh *et al*. showed that mouse Tregs can be transduced with a sodium/iodide symporter and thus enabled the radiolabelling of these Tregs with Technetium-99m pertechnetate (a radioactive substance) and subsequently visualised *in vivo* with a Single Photon Emission Computed Tomography (SPECT)/ Computerised Tomography (CT) imaging modality. After 24 hours post-injection into mice, these radiolabelled mouse Tregs were observed in the spleen (Sharif-Paghaleh et al. 2011). Human Tregs have also been radiolabelled in a similar manner to track their migratory pattern *in vivo* and is currently still under investigation within the host laboratory and unpublished work shows that human Tregs migrate to the transplanted human skin whereby the Tregs are likely to function to suppress the proliferation of Tresps (Jacinta Jacob, unpublished work). Whether, Treg EVs follow the same route as their parent Treg cells is unknown, hence, I aimed to label Treg EVs with eGFP-CD63 with the aim to track them *in vivo*, but due to time restrictions, I was not able to complete this part of the study. Nevertheless, given the CCR4 expression on these human Treg EVs, it does provide them with a potential property of homing to the human skin, similar to how their parent Tregs use CCR4 to home to the skin (Issa et al. 2012; Scottà et al. 2013).

6.5 Summary

In summary, human Treg EVs and Teff EVs were able to protect against alloimmune mediated inflammatory skin allograft damage by preventing $CD3^+$ T cells and $CD45^+$ immune cells infiltration and reducing proliferating keratinocytes as well as maintaining involucrin protein expression within the skin allograft. Labelling of Tregs with eGFP-CD63 or mCherry-CD63 viral particles are possible; however experimental optimisation is required to isolate $eGFP^+$ or $mCherry^+$ Treg EVs. If $eGFP^+$ or $mCherry^+$ Treg EVs can be isolated it can enable the researcher to track their migration *in vivo* and to understand their longevity.

Chapter 7
General Discussion and Future Work

Chapter 7 - General Discussion and Future Work

My data supports the idea that the release of EVs plays a key role in the functions of Treg cells. Given my findings it could be suggested that EVs released by Tregs used in clinical trials are influencing the clinical outcome, although this is highly speculative and requires further investigation. In clinical trials, Tregs are administered with drugs such as rapamycin, ATRA and IL-2 complex; whether these affect Treg release of EVs *in vivo* is not known. Interestingly, rapamycin regulates exosomes release. For example, Abdulrahman *et al*. showed that autophagy stimulation in neuronal cells in the presence of rapamycin inhibited exosomal prion release (Abdulrahman, Abdelaziz, and Schatzl 2018). Another study by Aung *et al*. demonstrated that 1μM, 5μM and 10μM concentrations of rapamycin inhibited exosomes release from B cell lymphoma cells, with 10μM concentration of rapamycin providing the most potent inhibition of exosomes release (Aung et al. 2011). It was suggested that rapamycin regulates MVB biogenesis and this might thus affect exosomes release (Aung et al. 2011). What is important to note from this study by Aung *et al*., is that these authors tested 1μM, 5μM and 10μM concentrations of rapamycin in affecting exosomes release. From their results it can be observed at 0μM to 10μM, the 1μM had a minimal effect on exosomes release compared to the 10μM concentrations of rapamycin used (Aung et al. 2011). However, the human Tregs used in this thesis were cultured with 100nM concentration of rapamycin ($1/10^{th}$ of the lowest concentration used by Aung *et al*. (Aung et al. 2011)). Whether 100nM concentration of rapamycin affects human Treg release of EVs is unknown, but human Tregs were cultured in the absence of rapamycin 48 hours prior to their activation for EVs release in case that rapamycin affected EV release from human Tregs.

ATRA, IL-2 and IL-2 complex (IL-2 in complexed with anti-IL-2) was demonstrated by Okoye *et al*. to regulate EV release from mouse Tregs. The presence of IL-2 complex on mouse Tregs resulted in a significant increase of exosomes whereas; the presence of ATRA resulted in a modest increase of exosomes release (Okoye et al. 2014).

The translation of Tregs therapy in the clinical setting was delayed for many years, which were exacerbated by concerns that Tregs were unstable (Zhou et al. 2009; Waldmann et al. 2014; Sawant and Vignali 2014; Shimon Sakaguchi et al. 2013;

Kleinewietfeld and Hafler 2013). Various studies have shown that under certain pro-inflammatory environments Tregs can convert into Th-17 like cells whereby they produce high levels of IL-17 (H. J. P. M. Koenen et al. 2008; X. O. Yang et al. 2008), thus Tregs can change from a regulatory cell to one that is immune stimulating. Therefore, further measures are required to increase and maintain the suppressive potency of these cells or to find an alternative or additional cell-free immunotherapy to ensure their stability for therapeutic use. Given our and other group's findings (Tung et al. 2018; Lesley Ann Smyth et al. 2013; Okoye et al. 2014; X. Yu et al. 2013; Aiello et al. 2017; Torri et al. 2017; Azimi et al. 2018), and the current interest in cell-free therapies, Treg EVs as a potential candidate for a novel cell-free therapy, alternative or combinatory therapy to whole Treg cell therapy is appealing. This thesis explored whether the use of these vesicles was possible in this setting. The results generated in this thesis demonstrated that human Treg EVs are functionally immunosuppressive perhaps due to their miRNA content, and can protect against transplant rejection, suggesting that Treg EVs may indeed have a future as a clinically applicable cell-free therapy for transplant patients.

Interestingly, the data exhibited herein showed that human Teff EVs, like the Treg EVs, were also immunosuppressive. Teff EVs can be both pro-inflammatory and immunosuppressive as described in section 1.7.7, highlighting the contrasting roles of Teff EVs. On the other hand, all 7 publications to date presenting Treg EVs have consistently and agreeably shown that Treg EVs are immunosuppressive (Tung et al. 2018; Lesley Ann Smyth et al. 2013; Okoye et al. 2014; X. Yu et al. 2013; Aiello et al. 2017; Torri et al. 2017; Azimi et al. 2018). Given this body of evidence, it favours Treg EVs over Teff EVs as an immunosuppressive agent for treatment of immune conditions such as diabetes and transplant rejection.

7.1 EVs treatment in clinical trials and future of Treg EVs for clinical use

Due to the complexity and complications of using whole cells for therapy, increasingly more research groups are pursuing the search for a cell-free vaccine or therapy. EVs are naturally-occurring biological particles which offer functions that may mirror certain characteristics of the parent cell and thus can potentially be used as an alternative or

combinatory therapy with whole cells for patients. My research mirrors these assumptions. EVs are more stable than cells and do not change according to their microenvironment, they can be stored in -80°C for 2 years without losing their potency (Ribeiro et al. 2013), and also for clinical trials, exosomes were stored at -80°C until use (Morse et al. 2005; Escudier et al. 2005; Dai et al. 2008; Besse et al. 2016). Furthermore, EVs are metabolically-inactive components and therefore do not require further expansion. In another report, EVs can be stored at -20°C for 6 months provided they are resuspended in non-toxic chemicals and EVs maintained their functions with minimal loss of efficiency (Barile et al. 2012). These reports together suggest that EVs can be stored as an 'off-the-shelf' product. Due to these collective attributes of EVs; cell-free product; GMP grade EVs/large scale manufacture possible; 'off-the-shelf' product; and ease of manipulation of EVs it promotes EVs as a potential therapy that circumvents many of the limitations of viable cells for therapeutic applications in healthcare medicine. However, the following question still needs addressing: can Treg derived EVs be isolated and used for clinical use as an 'off-the-shelf' product?

Large scale production of clinical grade GMP EVs is possible (Lamparski et al. 2002; Mendt et al. 2018). Although EVs research is generally in its infancy, exosomes has already reached phase I clinical trials (Morse et al. 2005; Escudier et al. 2005; Dai et al. 2008) and a phase II clinical trial (Besse et al. 2016). In all four of the clinical trials using exosomes described below (Morse et al. 2005; Escudier et al. 2005; Dai et al. 2008; Besse et al. 2016), exosomes were isolated using ultracentrifuge and sucrose gradient methods.

Morse *et al.* performed a phase I clinical trial testing the safety, feasibility and efficacy of using the cancer patients' own dendritic cells-derived exosomes (dexosomes) to treat non-small cell lung cancer (Morse et al. 2005). The dexosomes were loaded with MAGE tumour antigens with the aim to prime MAGE-specific T cell responses. How the authors decided on the dose of dexosomes to use was based upon how many MHC class II molecules were present on the dexosomes. The patients received 4 doses of exosomes vaccinations, which were given at 1 week intervals. The results showed that some of these non-small cell lung cancer patients that were immunised with the dexosomes vaccine had activation of immune effectors; MAGE-specific T cell responses and NK lytic activity; and stabilised the disease progression. This clinical trial concluded that

dexosomes therapy was generally well tolerated by the patients with no signs of serious toxicity observed (Morse et al. 2005).

Similar to Morse *et al*. (Morse et al. 2005), Escudier *et al*. also used patient-derived dexosomes to treat cancer patients, which also their doses depended upon the number of MHC class II molecules present on dexosomes (Escudier et al. 2005). The patients received 4 doses of exosomes vaccinations, which were given at 1 week intervals. These patients had stage III/IV metastatic melanoma and were treated with dexosomes loaded with MAGE 3 peptides. The results showed that one patient (from 15 patients) exhibited a partial response where there was depigmentation in the melanoma lesion. Additionally, two stable, one minor and one mixed response was also observed in some patients in the skin and lymph nodes regions. However, MAGE-3 specific $CD4^+$ and $CD8^+$ T cell responses was not observed in the peripheral of treated patients (Escudier et al. 2005).

In the two clinical trials in 2005 testing dexosomes by Morse *et al*. (Morse et al. 2005) and Escudier *et al*. (Escudier et al. 2005), the same authors had advanced their studies and recently published another clinical trial using dexosomes but this time in a phase II clinical trial (Besse et al. 2016). Besse *et al*. (Besse et al. 2016) used a more advanced dexosomes preparation treatment in comparison their first phase I clinical trials (Morse et al. 2005; Escudier et al. 2005). This dexosomes preparation, were also isolated using ultracentrifugation and sucrose gradient methods, but these dexosomes aimed to boost NK and T cell immune responses, by priming these dexosomes with IFNγ and loading them with MHC class I and II-restricted cancer antigens (IFNγ dexosomes). These IFNγ dexosomes were tested for the clinical benefit as a maintenance immunotherapy for non-small lung cell cancer patients (whom do not have tumour progression) after their induction chemotherapy treatment. In this study, 22 patients received IFNγ dexosomes injections; these patients received on median 7 IFNγ dexosomes injections with a range of 1-27 injections. These results indicated that IFNγ dexosomes did not induce cancer-specific T cell immune responses but it did boost NK cells activity against the tumour in these patients (Besse et al. 2016).

Patients' own ascites-derived exosomes have been tested for the clinical treatment of colorectal cancer. This phase I clinical trial by Dai *et al*. enrolled 40 patients, and the two treatment groups consisted of ascites-derived exosomes alone or ascites-derived

exosomes plus GM-CSF (Dai et al. 2008). Both treatment groups tolerated the therapeutic treatment and patients received 4 doses of exosomes vaccinations, which were given at 1 week intervals. Moreover, the patients whom received ascites-derived exosomes plus GM-CSF had elicited a more efficient and beneficial tumour-specific anti-tumour cytotoxic T lymphocyte response compared to the ascites-derived exosomes alone treatment group. Thus, the use of GM-CSF as an adjuvant significantly promoted the efficiency of ascites-derived exosomes therapy. These ascites-derived exosomes contained tumour-associated carcinoembryonic antigen and MHC molecules which the authors suggested that these may be recognised by epidermic APCs and thus indirectly stimulate T cell activation.

Furthermore, there are multiple ongoing clinical trials registered on http://clinicaltrials.gov which are using exosomes either as a therapy or as a biomarker discovery source. For example, ClinicalTrials.gov Identifier: NCT02138331 aimed to test MSC-derived microvesicles and exosomes therapy on islet β-cells of the pancreas, in the aim to reduce inflammation and treat or manage type I diabetes mellitus. In another clinical trial: NCT03384433, it aims to administer allogeneic MSC exosomes, enriched in miR-124 to treat patients with acute ischemic stroke-associated disability. Clinical trials: NCT03102268; NCT01344109; NCT02702856 and NCT03562715 aims to detect exosome-associated miRNA, ncRNA or other RNA molecules signatures that may be used as diagnostic or prognostic markers in cancers and various other health conditions. Clinical trial: NCT02327403 aims to test urine extracellular vesicles from kidney transplant patients to assess B7-1 (CD80) derived from APCs.

Taking the above together; this thesis findings; GMP isolation of EVs; the published and the ongoing clinical trials that used or uses EVs, highly supports that Treg EVs have the potential to reach clinical trials and thus likely to be a favourable agent to use in the setting of autoimmune diseases such as diabetes to suppress the immune responses. Current commercial biotechnology-based companies are developing EVs for use in clinical trials and GMP-grade isolation of EVs is commonplace. GMP-grade ExoQuick™ (ExoQuick-CG™) is available for isolating EVs in a GMP manner for clinical use. Therefore, the likelihood of EVs use in the clinic setting is highly feasible. As described above, DC EVs are used for treating various cancers due to the immune promoting

properties, whereas Treg EVs would be immune suppressing and thus likely unsuitable to treat cancer patients.

7.2 Improving the use of Treg EVs for clinical use

Theoretically, GMP-grade isolation of human Treg EVs should be possible. But, should Treg EVs be used alone or together with Tregs? This is a question that remains open. Given that Treg EVs are not as potent as parent Tregs, it could be suggested to initially be used as a combination therapy after which immunological tolerance of the transplant can be maintained by the treatment of cell-free Treg EVs. In addition, extensive research is currently ongoing to manipulate EVs to carry certain drugs, miRNA or other biological components within them and these vesicles may be engineered to target specific cells to deliver drugs and other biological material.

I have shown in this thesis that Treg EVs alone modulates cytokine production by BM-DCs (Chapter 3) and by Tresps (Chapter 4), whether Treg EVs together with other agents such as rapamycin, ATRA, IL-2 or IL-2 complex or with whole Treg cells can further modulate cytokine profile production by Tresps is worth further investigations. Given that cytokines potently affect immune cells, it can be envisaged that Treg EVs used alone or in combination with other agents can be used in favour to suppress pro-inflammatory cytokines whilst concurrently promoting anti-inflammatory cytokine production. Hence, with this aim it can potentially benefit the clinical outcome of transplant patients. In the next subsections I will discuss the aforementioned.

7.2.1 EVs combined with rapamycin or other agents treatment

The use of immunosuppressive drugs together with EVs has been shown to promote transplant tolerance *in vivo*. A study by Li *et al*. showed that immature DC exosomes together with rapamycin promoted heart allograft tolerance in mice (X. Li et al. 2012). Moreover, in another study by Peche *et al*., these authors demonstrated that donor exosomes together with short-term immunosuppression induced cardiac transplant tolerance in fully MHC-mismatched rats (Peche et al. 2006). These studies are further detailed in section 1.7.10.

Therefore, taken the above, engineering EVs to carry specific drugs to the allograft may clinically benefit transplant recipients. Furthermore, the use of rapamycin or other immunosuppressive drugs in culturing Tregs for EV release or using Treg EVs in combination with immunosuppressive drugs may favour the clinical outcome for transplant patients and warrants further investigation.

7.2.2 EVs combined with IL-2 modulate cytokine production

Various miRNAs can induce Tregs (section 5.4.4); similarly, EVs have also been reported to regulate Tregs. EVs have been described by various groups to promote the induction of Tregs (Song et al. 2016; G.-J. Wang et al. 2008; Wieckowski et al. 2009), or conversely, can reduce the frequency of Tregs (Rao et al. 2016; Kimura et al. 2018). Whether Treg EVs induce Tregs is unknown. However, a study in 2012 by Wahlgren *et al.* had shed some light into human $CD3^+$ T cell derived exosomes and their ability to modulate autologous cells (Wahlgren et al. 2012). Wahlgren *et al*. isolated exosomes from activated $CD3^+$ T cells, and together with IL-2 treated resting $CD3^+$ T cells and observed their responses. The exosomes plus IL-2 treatment was able to stimulate proliferation in autologous resting $CD3^+$ T cells, more efficiently than exosomes or IL-2 treatment alone. Importantly, the use of IL-2 alone, exosomes alone or exosomes plus IL-2 results in different cytokine production profiles from the target $CD3^+$ T cells. Namely, when the $CD3^+$ T cells were treated with IL-2 alone or exosomes alone, IL-4 was downregulated whilst treatment with exosomes plus IL-2 upregulated IL-4 production. With IL-2 alone treatment, IL-6 was downregulated whereas with exosomes alone treatment, it was upregulated by 0.4 folds and with exosomes plus IL-2 treatment, IL-6 was upregulated by 4 folds. Furthermore, IL-10 was also affected. IL-2 alone and exosomes plus IL-2 treatments both upregulated IL-10 production by 0.5 folds, whereas exosomes alone treatment, IL-10 was downregulated by 2 folds (Wahlgren et al. 2012). Thus, whether EVs are injected alone or with IL2 can significantly modify the cytokine microenvironment.

7.2.3 EVs combined with Tregs in transplantation

Ma *et al*. showed that the combined therapy of immature DC exosomes with donor antigen-specific Tregs induces liver transplant tolerance in rats (Ma et al. 2016). In these treated rats, the infiltrating cells in the allograft were reduced and the recipient immune responses were inhibited in a donor-specific manner. These authors suggested that immature DC exosomes may have promoted the proliferation of Tregs within the allograft, which as a result may overall enhance the regulatory effects of the treatment (Ma et al. 2016). Noteworthy, immature DC exosomes together with donor antigen-specific Tregs induced transplant tolerance without the need for immunosuppressive drugs (Ma et al. 2016). Whether a similar approach can be used with human Treg EVs together with donor-antigen specific Tregs to induce transplant tolerance in patients would be interesting to investigate.

7.3 Modifying Treg EVs for therapy

What is also achievable is the engineering of EVs to package specific miRNAs (Luan et al. 2017). miRNAs have been linked to inducing or promoting the expansion of Tregs (section 5.4.4), and in the context of transplantation, tipping the balance in favour of a higher frequency of Tregs than Teffs leads to a better outcome in transplant patients. Indeed, the clinical trial: NCT03384433, uses exosomes loaded with miRNA to treat patients with acute ischemic stroke-associated disability. As reviewed by Jeker and Bluestone, miRNAs are key biological molecules that regulate T cell differentiation and function and more is being discovered about how miRNA shapes the immune system (Jeker and Bluestone 2013). Many miRNA functions are still being fully elucidated and many of the miRNAs found in the Treg EVs have been linked to various aspects of inhibiting T cell activation and their proliferation or inducing Treg expansion. This further supports my finding that Treg EVs not only are immunosuppressive, but their miRNA cargo is suggested to be selective possibly to enhance a regulatory effect in the target cells. Collectively, taking the above publications together with Chapter 5 Discussion (section 5.4), it provides support that designing and engineering Treg EVs to carry specific miRNAs that inhibit pro-inflammatory cytokine production and/or promote Tregs expansion; and using these engineered Treg EVs alone or in combination

with rapamycin, ATRA, IL-2, IL-2 complex or other agents may have potential in the clinic for transplant patients.

Which miRNAs could be targeting to Treg EVs? I found that mouse Treg EVs transferred miR-142-3p and miR-150-5p into BM-DCs. These miRNAs have previously been reported to modulate DC cytokine profiles (section 3.4.4). Indeed, mouse Treg EVs reduced IL-6 whilst increased IL-10 production levels by BM-DCs, perhaps to prevent a pro-inflammatory microenvironment and instead promote an anti-inflammatory milieu. Exclusive miRNAs which were absent in Tregs but enriched in human Treg EVs included; miR-369-3p, miR-376c-3p and miR-195-3p, which were predicted to target the 3' UTR regions of IFNγ, IL-2 and IL-6 mRNA. I also found that in the human miRNOME, miR-99a-5p were found higher expression levels in Treg EVs than Tregs and not present in Teff EVs; and miR-150 were found in higher expression levels in Treg EVs than Teff EVs. A study by Warth *et al*. demonstrated that miR-99a and miR-150 work in unison to inhibit the Th17-promoting mTOR activity to promote Treg differentiation (Warth et al. 2015). Furthermore, miR-100 which was found in the human Treg EVs and not Teff EVs, was also described by Warth *et al*. to promote Tregs and inhibit Th17 differentiation (Warth et al. 2015)

More recently, there is growing interest in the use of chimeric antigen receptor (CAR) T cell exosomes for immunotherapy against cancers instead of CAR T cells due to the cell treatment-related toxicities (X.-J. Tang et al. 2015). CARs are engineered recombinant receptors that implement a specificity of a monoclonal antibody onto a cell with the aim of the CAR cell to bind specific markers found on target cells to modulate them. Whether CAR Tregs release CAR EVs has so far not been demonstrated. However, in theory if CAR Treg EVs are possible it can be anticipated that these CAR EVs will be designed and engineered to be allospecific to the transplant graft and thus CAR EVs and their cargo may be more efficiently delivered to the allograft to suppress immune cells and thus inhibit transplant rejection.

Nonetheless, CAR exosomes have been assessed as a drug delivery mechanism to treat mantle cell lymphoma (Bao et al. 2017). This study by Bao *et al.* used CAR engineered exosomes that exhibited a membrane fused anti-CD19 single chain variable fragment (scFv) which were designed to package and carry doxorubicin drug to be delivered to mantle cell lymphoma cells *in vitro* and *in vivo* (Bao et al. 2017). The results

demonstrated that treatment of mantle cell lymphoma cells with CAR exosomes carrying doxorubicin increased toxicity more efficiently than free doxorubicin alone. Thus, EVs derived from Tregs may also have the capacity to package and deliver immunosuppressive drugs to targeted cells, namely, T cells and DCs, the main culprit cells in driving transplant rejection.

In conclusion, the scope of designing and engineering Treg EVs for their application in the clinical setting to treat transplant rejection is undeniably promising given the collective data presented in this thesis and all the mentioned publications that provide support that Treg EVs immunotherapy may one day, in the future, benefit transplant patients. Future studies are however still required to investigate where EVs migrate to once it is administered into a patient as well as their safety. For example, would they induce wide spread immunosuppression; something that could be addressed by creating antigen-specific EVs. Although I have shown in humanised mouse models of transplantation that human Treg EVs function *in vivo* to protect against allograft damage, whether the same occurs in humans remains to be elucidated. Given that exosomes are being used in clinical trials, the next step in my research would be to define protocols to isolate GMP compatible Treg EVs and to move these exciting structures into the clinic.

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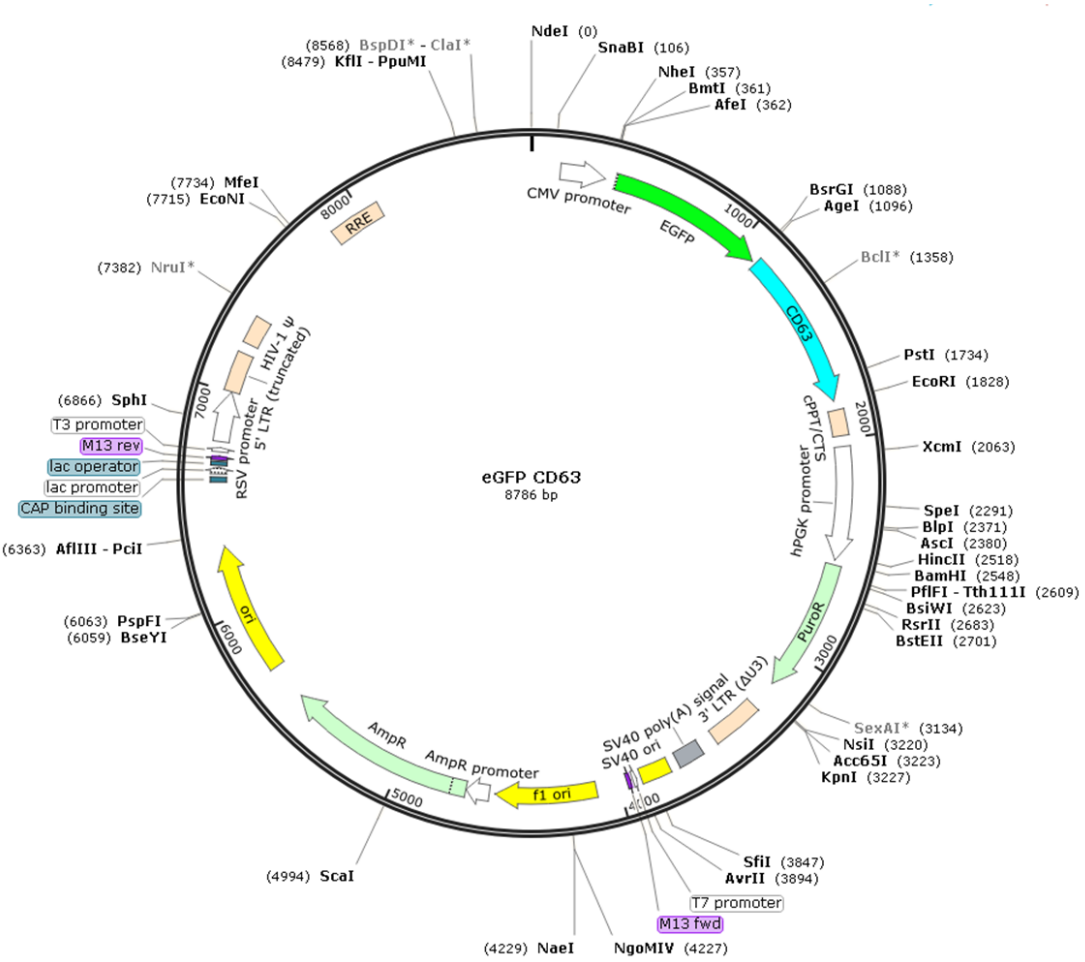
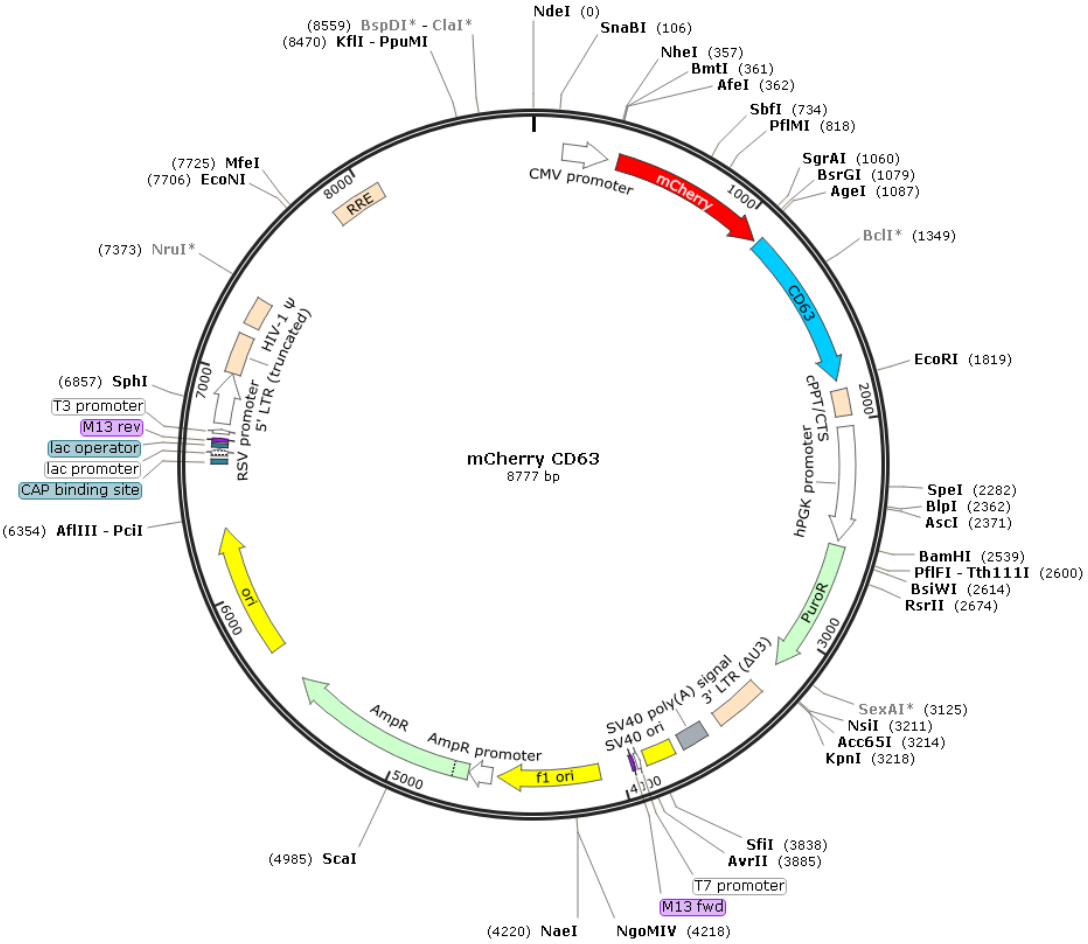
Appendices



Appendix 1- Plasmid map of eGFP-CD63



Appendix 2- Plasmid map of mCherry-CD63

Appendix 3- IFNγ mirSVR and PhastCons scores

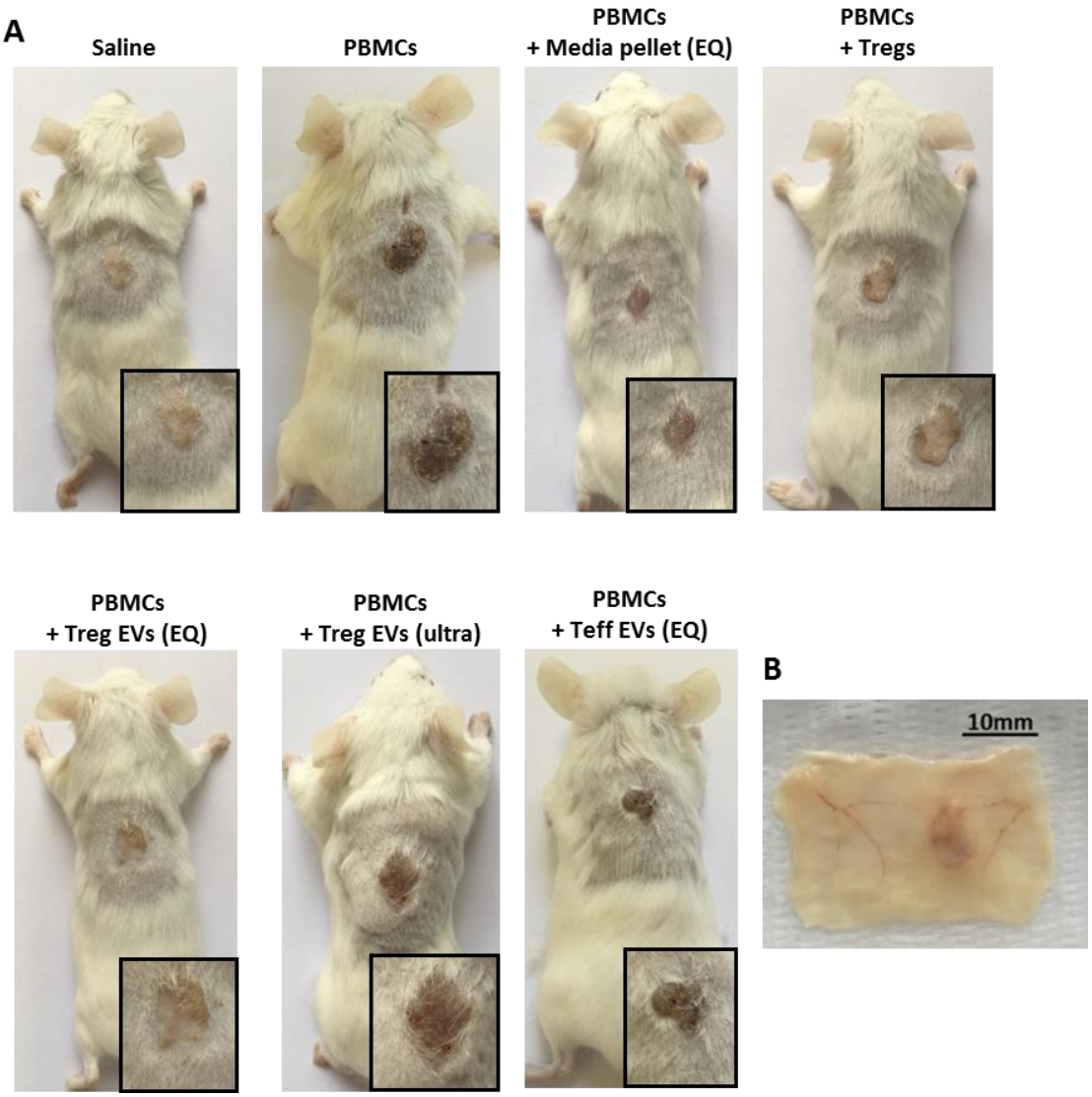
Specific alignments of each miRNA to IFNγ 3' UTR of mRNA and associated miSVR and PhastCons scores are indicated on the right.

Appendix 4- IL-2 mirSVR and PhastCons scores

Specific alignments of each miRNA to IL-2 3' UTR of mRNA and associated miSVR and PhastCons scores are indicated on the right.

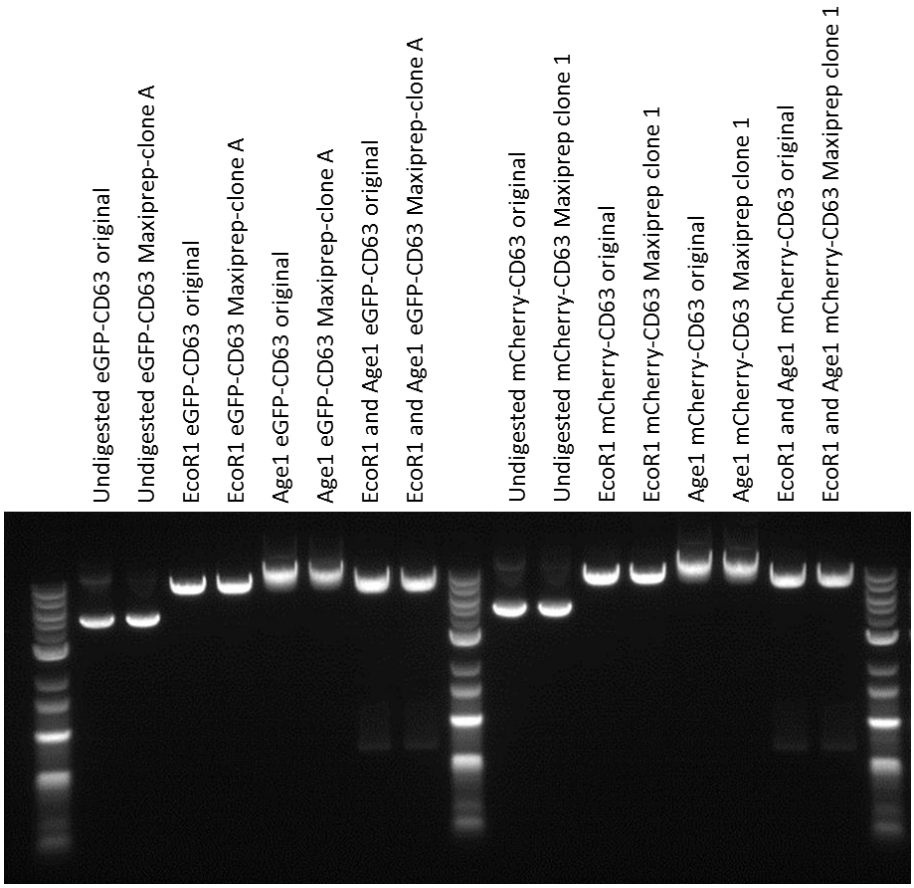
Appendix 5- IL-6 mirSVR and PhastCons scores

Specific alignments of each miRNA to IL-6 3’ UTR of mRNA and associated miSVR and PhastCons scores are indicated on the right.



Appendix 6- Macroscopic analysis of human skin transplanted mice with various treatments

BRG immunodeficient mice were transplanted with a section of human skin which was derived from healthy donors with written consent. After 35 days, the mice received saline or allogeneic CD25$^-$ PBMCs, in the absence or presence of autologous 1x10^6 Tregs, 50x10^6 Tregs-derived EVs isolated by ExoQuick-TC™ (EQ), 50x10^6 Tregs-derived EVs isolated by ultracentrifugation (ultra), 50x10^6 Teffs-derived EVs isolated by EQ or the pellet resulting from media alone mixed with EQ (media pellet EQ). At the end point of study, human skin allografts were assessed **(A)** *macroscopically and* **(B)** *on the underside; displayed is a representative image of the human skin allograft on the ventral position. Scar bar indicates 10mm.*



Appendix 7- Maxiprep restriction analysis results

To prepare plasmid DNA of eGFP-CD63 and mCherry-CD63, maxipreps were processed and restriction analysis using EcoRI-HF® and AgeI-HF® enzymes performed on the selected clones to confirm the correct DNA was purified.

Related Publications

*Joint first authors

Regulatory T-cell derived extracellular vesicles modify dendritic cell function

***Sim L. Tung**, *Dominic A. Boardman, Monica Sen, Marilena Letizia, Qi Peng, Nicole Cianci, Laura Dioni, Leo M. Carlin, Robert Lechler, Valentina Bollati, Giovanna Lombardi and Lesley A. Smyth.

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Treg therapy in transplantation: a general overview

*Marco Romano, ***Sim Lai Tung**, Lesley Ann Smyth, Giovanna Lombardi

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Regulatory T cell-derived exosomes: possible therapeutic and diagnostic tools in transplantation

Akansha Agarwal, Giorgia Fanelli, Marilena Letizia, **Sim Tung**, Dominic Boardman, Robert Lechler, Giovanna Lombardi, Lesley Smyth

Frontiers in Immunology (2014). Volume 5, Article 555, Pages 1-7

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MicroRNAs affect dendritic cell function and phenotype

Lesley Smyth, Dominic Boardman, **Sim Tung**, Robert Lechler, Giovanna Lombardi

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Regulatory T cell-derived extracellular vesicles modify dendritic cell function

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Regulatory T cells (Treg) are a subpopulation of T cells that maintain tolerance to self and limit other immune responses. They achieve this through different mechanisms including the release of extracellular vesicles (EVs) such as exosomes as shown by us, and others. One of the ways that Treg derived EVs inhibit target cells such as effector T cells is via the transfer of miRNA. Another key target for the immunoregulatory function of Tregs is the dendritic cells (DCs). In this study we demonstrate directly, and for the first time, that miRNAs are transferred from Tregs to DCs via Treg derived EVs. In particular two miRNAs, namely miR-150-5p and miR-142-3p, were increased in DCs following their interaction with Tregs and Treg derived exosomes. One of the consequences for DCs following the acquisition of miRNAs contained in Treg derived EVs was the induction of a tolerogenic phenotype in these cells, with increased IL-10 and decreased IL-6 production being observed following LPS stimulation. Altogether our findings provide data to support the idea that intercellular transfer of miRNAs via EVs may be a novel mechanism by which Tregs regulate DC function and could represent a mechanism to inhibit immune reactions in tissues.

Naturally occurring, thymus derived $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) play a fundamental role in the maintenance of immunological tolerance, preventing autoimmunity, as well as limiting immune responses[1–3]. Although they can act directly on effector T cells (Teffs), Tregs also target the orchestrators of the immune response, the dendritic cells (DCs)[4–7]. Recently, Yan *et al*. have shown, using intravital microscopy, that endogenous murine Tregs form prolonged contacts with injected LPS-treated DCs in lymph nodes, which was enhanced in the presence of interleukin (IL)−2[8]. These authors also measured the interaction times between antigen specific Teffs and DCs in the absence or presence of IL-2 pre-treated polyclonal Tregs. They found that DC-Teff cell contact time was significantly reduced in the presence of Tregs[8]. This *in vivo* data confirmed previous *in vitro* observations[6,7] whereby Tregs, not treated with IL-2, clustered around DCs inhibiting Teff-DC interactions. The molecular bases of DC-Treg interactions was also recently revisited by Chen *et al*., using super resolution imaging[9]. These authors showed that the interaction between these two cell types was dependent on strong LFA-1-ICAM-1 binding by Tregs, as previously described[6]. This altered the cytoskeleton of DCs leading to sequestering of a molecule involved in the immunological synapse (IS), called Fascin 1, to the contact area between Treg and DC. In doing so, Fascin1 availability for IS formation between Teffs and DCs was reduced[9].

This is just one way by which Tregs have been found to modify DCs[5]. Several mechanisms, IDO production, CD39/CD73 expression induced adenosine production and CTLA-4-facilitated removal of co-stimulatory molecules, CD80 and CD86, from the membrane of the DCs[10,11] have all been attributed to how Treg modify DCs leading to reduced T cell responses. Regardless of the mechanism used, exposure to Tregs alters the ability of DCs to activate T cells and modifies cytokine profile[8,12–16]. In several *in vitro* studies Treg-treated DCs displayed reduced expression of CD80 and CD86[6] as well as CD38 and CD83, in the human setting, compared to immature

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and mature DCs[17]. In addition they release inhibitory cytokines including IL-10, transforming growth factor (TGF)-β and IL-35 and have reduced IL-12 production following TLR activation[5,17].

We, and others, have described the release of immune inhibitory exosomes from murine Tregs[18–20]. Given that the isolation protocols used to isolate exosomes in these studies may also include other small vesicles, as described by Kowal *et al.* the term extracellular vesicles (EVs) will be used in this manuscript instead of exosomes[21]. These structures have been shown to facilitate the intercellular transfer of small non-coding RNAs, microRNAs (miRNAs), including Let 7b, Let 7d, miR-150 and miR-155 from Tregs to Teffs[18,19]. Importantly miRNAs in Treg EVs were functional following transfer, repressing target mRNAs in the Teffs both *in vitro* and *in vivo*[19]. Whether miRNAs are transferred by Treg EVs to DCs has yet to be addressed, however Okoye *et al.* observed that fluorescent oligonucleotide duplexes (FL-dsRNA) expressed in murine Tregs could be acquired by DCs following co-culture suggesting that the transfer of miRNAs between these cells is a possibility[19]. In addition, the intercellular transfer of miRNAs containing EVs from murine T cells to APCs via vesicles has been observed[22]. Recently, CD8 suppressor murine T cells were found to release EVs containing miR-150, which modified macrophage function[18,23].

A better understanding of the contribution of Treg derived EVs to maintaining tolerance is warranted. Given that DCs and Tregs interact *in vivo* and that the miRNAs expressed in DCs play a key role in modulating their function, specifically their cytokine production and their phagocytic capacities[24], we investigated whether miRNAs present in Treg derived EVs are acquired by DCs and whether this affects the function of these cells.

Results

Murine Tregs and their EVs modulate DC cytokine production. DCs are a major target of $CD4^+CD25^+Foxp3^+$ Tregs. One of the consequences of this cellular interaction is the acquisition by DCs of a 'tolerogenic' phenotype[5,6]. The hypothesis that was tested in this study is whether EVs released by Tregs contribute to the 'tolerogenic' effect of DCs. To test this possibility a very well characterised murine Treg line, generated by stimulating B6 $CD4^+CD25^+Foxp3^+$ cells *in vitro* with allogenic BALB/c DCs was used. This Treg line has direct allospecificity for BALB/c MHC class II molecule, $I\text{-}A^d$, antigens[25–27] and will be referred to as dTregs throughout the manuscript. Phenotypic analysis of the BALB/c-specific Tregs showed that they expressed CD4, the IL-2 receptor α-chain (CD25) and the lineage-defining transcription factor FoxP3, as well as CTLA-4, CD73 and GITR (Fig. 1A). These cells were also highly suppressive; proliferation of B6 $CD4^+$ responder T cells (Tresp) upon stimulation with allogeneic BALB/c APCs was significantly decreased ($p = 0.0092$) by the presence of dTregs at a 1:1 Treg:Tresp ratio (Fig. 1B). In addition, these Tregs interacted with BALB/c BM-DCs. Real time imaging of BALB/c DCs co-cultured with CFDA (green) labelled BALB/c-specific Tregs indicated that dTregs formed conjugates with BALB/c derived bone-marrow (BM)-DCs (Fig. 1C). Before evaluating the effect of Treg derived EVs, we confirmed that by co-culturing Tregs with BM-DCs, the expression of CD80 and MHC class II on DCs was reduced as well as the production of the pro-inflammatory cytokine IL-12 induced by LPS-mediated activation. These results confirmed previous findings that dTregs can modify DC phenotype and function (Fig. 1D).

Next we assessed whether dTreg derived EVs modified DCs in a similar manner. As expected, Tregs produced EVs of around 100 nm following TCR activation (Fig. 2A and Supplemental Fig. 3) which were found to be 'acquired' by BM-DCs upon co-culture (Fig. 2B). In contrast to Tregs, dTreg derived EVs did not influence the expression of the co-stimulatory molecule CD80 (Fig. 2C) by BM-DCs. However, we did observe that following LPS activation, IL-6, but not TNFα, was significantly reduced in Treg derived EVs treated BM-DCs, as compared to controls (Fig. 2C). Interestingly, we also observed that IL-10 release was significantly increased (Fig. 2C) in LPS activated DCs that had been co-cultured in the presence of Treg-derived EVs but no changes were observed for TNFα. This was not due to the presence of IL-10 in the EVs (data not shown).

Taken together, our data suggests that both dTregs and their EVs modify DCs following interaction with the latter, predominantly affecting cytokine production, although EVs and Tregs affect BM-DCs in a different manner.

miRNAs present in Tregs were increased in BM-DCs following co-culture. Having established that dTregs and their released vesicles interacted with and modified BM-DCs, we addressed whether the changes in the phenotype and function of BM-DCs was mediated at least in part by miRNAs originating from Tregs. A microarray screen was performed to identify differentially expressed miRNAs in dTregs and BM-DCs. Of 319 mouse miRNAs (miRBase 18 definition) analysed, 6 were expressed at significantly higher levels in the Tregs compared to the DCs ($p < 0.05$; fold-change > 1.5). Identified miRNAs included miR-150-5p, miR-182-5p, miR-125b-5p, miR-3096-5p, miR-3096b-5p and miR-96-5p (Fig. 3A and Supplemental Fig. 1) and their differential expression was confirmed by qPCR (Fig. 3B).

Following TCR activation, T cells miRNAs profiles are modified with some miRNAs being downregulated[28,29] whilst others are increased[29]. To assess how expression of the aforementioned miRNAs profiles were altered following activation, dTregs were stimulated with anti-CD3/CD28 beads for 24 hours and then analysed for miRNA expression by qPCR. We observed that miR-150-5p ($p = 0.02$) and miR-125b-5p ($p = 0.052$) were both down-regulated upon stimulation (Fig. 3C) however miR-182-5p was not affected upon stimulation ($p = 0.54$).

To assess whether intercellular transfer of the aforementioned miRNAs might occur between Tregs and DCs, BALB/c BM-DCs were co-cultured with dTregs at a 1:1 ratio for 24 hours, $CD4^-CD11c^+$ DCs were isolated by FACS sorting (purity > 99%, data not shown) and miRNAs present in the DCs were evaluated by qPCR. DCs co-cultured with dTregs contained significantly more miR-125b-5p ($p = 0.022$) and miR-150-5p ($p = 0.039$) compared to DCs cultured alone (Fig. 4A and B, respectively). However, no change in the presence of miR-182-5p ($p = 0.54$) was observed (Fig. 4C). These observations demonstrate that certain miRNAs, which are highly expressed in Tregs, are present at higher levels in BM-DCs following co-culture, suggesting that intercellular miRNA transfer may have occurred.

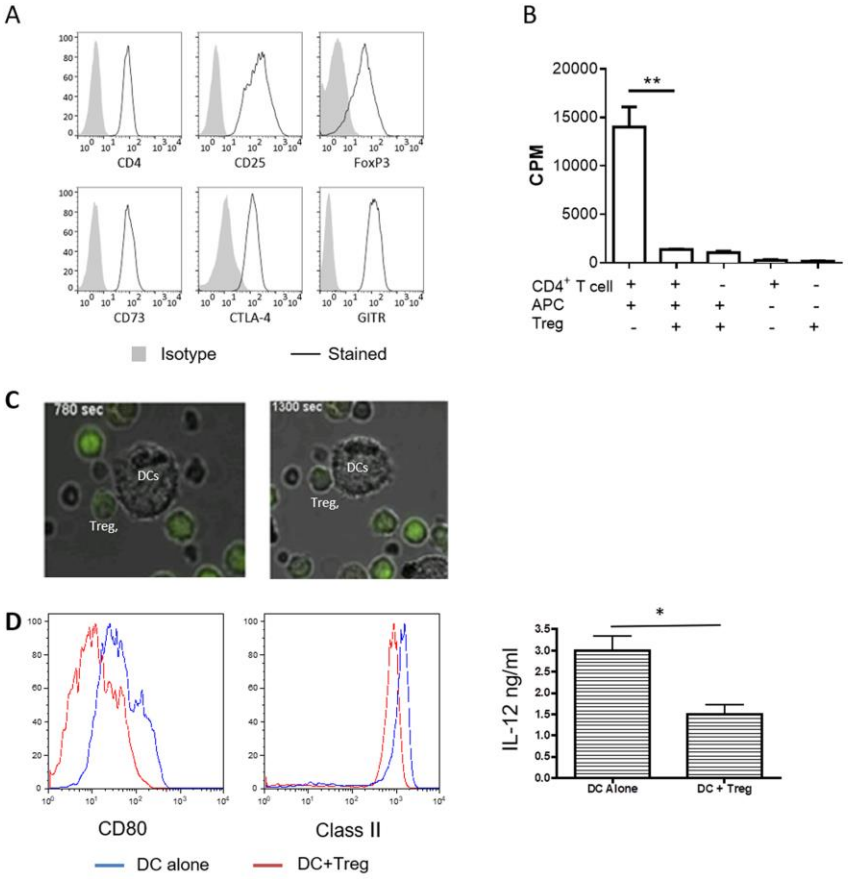

Figure 1. Murine $CD4^+CD25^+$ Tregs induce a tolerogenic phenotype in dendritic cells following co-culture. (**A**) Flow cytometry plots showing the expression of typical Treg markers by $CD4^+CD25^+$ T cells from a previously established cell line (black line). Isotype control staining is shown in solid grey. Data shown is representative of 8 experiments. (**B**) Suppressive capacity of $CD4^+CD25^+$ dTregs. B6 $CD4^+$ T cells were stimulated with BALB/c APCs in the presence or absence of an equal number of dTregs. Co-cultures were performed for 3 days and proliferation was measured by ^{3}H-thymidine incorporation. Data are representative of 5 experiments. (**C**) Confocal microscopy image of DCs co-cultured with $CD4^+CD25^+$ dTregs (green). Data are representative of 2 experiments. (**D**) Activation capacity of BM-DCs following co-culture with dTregs. BALB/c BM-DCs were co-cultured alone (blue line) or with dTregs (red line) for 24 hours to induce a tolerogenic phenotype. $CD11c^+CD4^-$ DCs were isolated by cell sorting and activated with 100 ng/mL LPS overnight. MHC class II ($I\text{-}A^d$) and CD80 expression was measured by flow cytometry and IL-12 (ng/ml) was measured by cytokine-specific ELISA. Data represents 3 individual experiments that were performed in duplicates. Statistical significance was determined using a two-tailed paired Student's t-test where $*p < 0.05$ and $**p < 0.01$. CPM = counts per minute.

Treg EVs contain specific miRNAs. To assess whether the increase in miRNAs seen in the BM-DCs following co-culture was due to the delivery of these RNA species via Treg EVs, we analysed the total RNA contents of EVs derived from dTregs and from a control $CD4^+FoxP3^-$ T cell line (Fig. 2A supplemental). As expected, miRNA extracted from EVs contained no ribosomal RNA, as evident by the absence of 18 S and 28 S peaks (Supplemental Fig. 4)[30–32] whilst conversely, RNA isolated from the two T cell preparations did contain these larger ribosomal RNA species, as seen on the electropherogram (Supplemental Fig. 4). MiRNAs present in the EVs were profiled using a QuantStudio 12 K Flex Real time PCR system (Supplemental Table 1). A Volcano plot (Fig. 5A) was produced to select miRNAs characterized by more than ± 2-fold differences between Treg and control $FoxP3^-$ T cell EV content (Log2 (Fold Change) > 1 or < −1) with a log10(p-value) > 1.301. Comparing the miRNAs profiles of EVs derived from dTreg and control T cells, we observed that many miRNAs were common

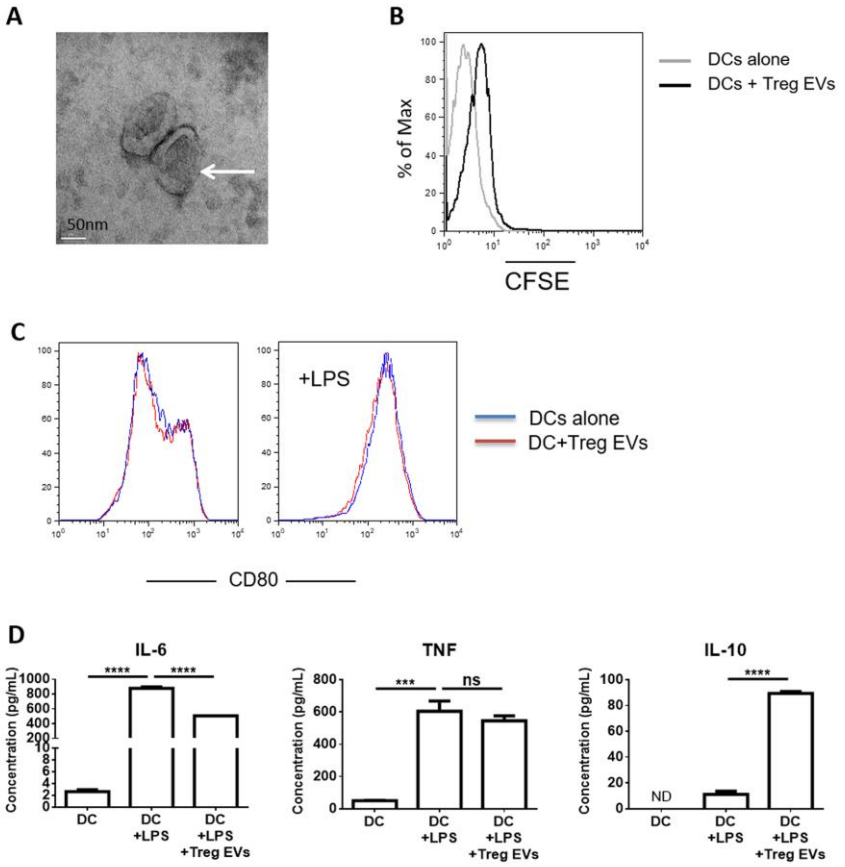

Figure 2. Murine Treg derived EVs are acquired by dendritic cells and alter cytokine production. (**A**) Electron micrograph image of EVs released from dTregs activated by plate-bound αCD3/CD28 antibodies isolated by ultracentrifugation. Scale bar indicates 50 nm. Representative image from 3 experiments is shown. (**B**) Treg cells were labelled with CFSE and activated by plate bound αCD3/CD28 antibodies for 24 hours. Supernatant was collected, cells and cell debris depleted and EVs isolated via ultracentrifugation (Treg EVs). BM-DCs were cultured alone or co-cultured with $CFSE^+$ Treg EVs for 24 hours. Flow cytometry histogram plot showing CFSE expression levels of DCs cultured alone (grey) and DCs co-cultured with $CFSE^+$ Treg EVs (black). Data is representative of 3 independent experiments. (**C**) BM-DCs were co-cultured alone (blue line) or with Treg derived EVs (red line) in the presence of 100 ng/mL LPS and CD80 expression was measured by flow cytometry after 24 hours. (**D**) IL-6, TNF and IL-10 cytokine production by BM-DC (DC+LPS), BM-DCs pre-treated with Treg EVs for 24 hours (DC + LPS + Treg EVs) and untreated BM-DCs (DCs) following LPS activated (24 hours) was assessed by cytometric bead array and flow cytometry. Data represents 2 independent experiments that were performed in technical triplicates, mean + SEM is shown. Statistical significance was determined using One-way ANOVA and Tukey's multiple comparison test. ***$p < 0.001$, ****$p < 0.0001$, NS = not significant and ND = not determined.

in both EVs (Fig. 5A and Supplemental Table 1). However, several miRNAs were differentially detected (Fig. 5A and Supplemental Table 1) including three identified miRNAs, miR-142-3p, miR-150-5p and miR-384-5p. This was further confirmed by qPCR (Fig. 5B). For example, miR-384-5p was present at significantly higher levels in EVs isolated from the $CD4^+Foxp3^-$ cell line ($p = 0.0073$) whilst miR-142-3p was present at significantly higher levels in Treg EVs (Fig. 5B, $p = 0.0319$). Interestingly, miR-150-5p, a miRNA shown previously to be present in EVs isolated from human $CD4^+$ T suppressor cells[33], was also found in EVs isolated from the murine dTreg line used in this study (Fig. 5B, middle panel, $p = 0.0381$).

As mentioned, we detected higher levels of miR-142-3p in the Treg EVs compared to control EVs. This miRNA was not identified as being significantly differentially expressed ($p = 0.0688$; fold change = 0.231) between dTregs

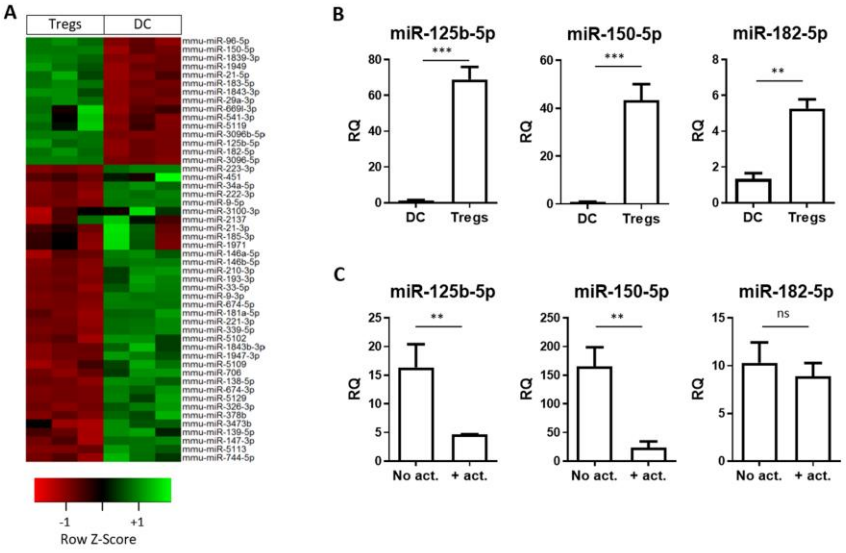

Figure 3. Murine $CD4^+CD25^+$ dTregs express high levels of specific miRNAs compared to $CD11c^+$ BM-DCs. (**A**) Microarray heat map diagram showing a two-way unsupervised hierarchical clustering of miRNAs by $CD4^+CD25^+$ dTregs and BM-DCs microRNA. (**B**) Bar graphs (mean+SEM) showing the relative quantity (RQ) of miR-125b-5p, miR-150-5p, and miR-182-5p in BM-DCs and dTregs, as measured by qPCR and normalised relative to RNU6-2 and SCARNA17. Data represents 2 individual experiments that were performed in duplicates. Statistical significance was determined using a two-tailed paired Student's t-test where $^{**}p < 0.01$ and $^{***}p < 0.001$. (**C**) Bar graphs (mean + SEM) showing the RQ of indicated miRNAs by Tregs immediately before (no act) and after (+act) polyclonal stimulation with anti-CD3/CD28 beads, as measured by qPCR and normalised relative to RNU6-2 and SCARNA17.

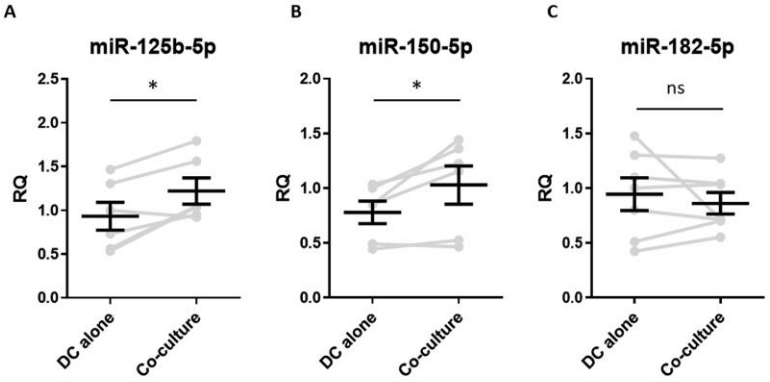

Figure 4. BM-DCs obtain dTreg miRNAs following co-culture with Tregs. Bar graphs showing the relative quantity (RQ) of (**A**) miR-125b-5p, (**B**) miR-150-5p, and (**C**) miR-182-5p expressed by FACS sorted $CD11c^+CD4^-$ DCs BM-DCs following co-culture with dTregs, as measured by qPCR and normalised relative to RNU6-2 and SCARNA17. DC-alone, represents BM-DCs cultured alone and FACS sorted. Data from 6 individual experiments is shown; each grey line represents an individual experiment and the pooled mean ± SEM of these experiments is shown in black. Statistical significance was determined using a two-tailed paired Student's t-test where $^*p < 0.05$. ns = not significant.

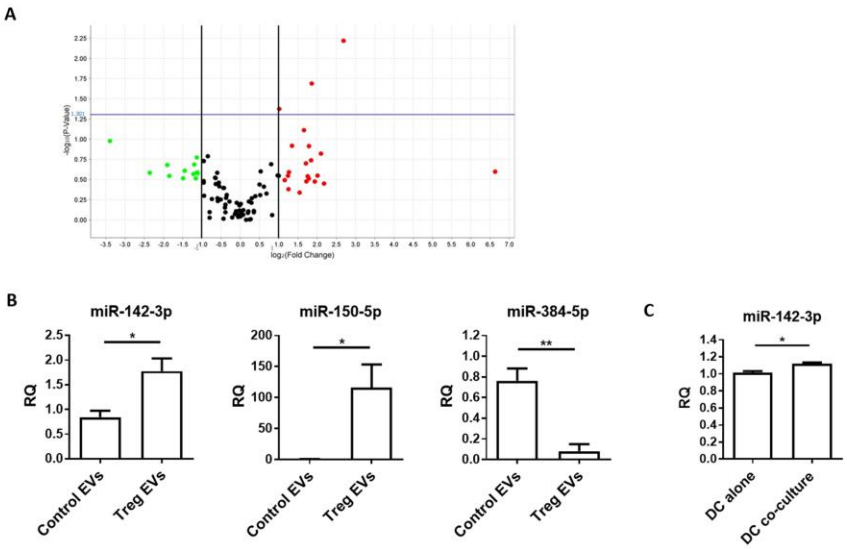

Figure 5. Murine $CD4^+CD25^+$ Treg EVs contain different miRNA compared to control T cell EVs. (**A**) dTreg and control $FoxP3^-$ T cell EVs (n = 3 per group) isolated by ExoQuick-TC were lysed and total RNA was purified and assessed using NanoDrop™ spectrophotometer and Agilent 2100 Bioanalyser. EVs miRNA was reverse transcribed into cDNA and pre-amplified before miRNA detection using QuantStudio™ 12 K Flex Real-Time PCR System with the OpenArray® Platform. The volcano plot shows the relation between the p-value and the log fold change between $FoxP3^-$ T cell and Treg EV miRNA expression levels. The blue line indicates the inverse $\log_{10}$ of the p-value = 0.05. (**B**) Bar graphs (mean + SEM) showing the relative quantity of miR-142-3p, miR-150-5p, and miR-384-5p by control T cell EVs (Control EVs) and Treg EVs (Treg EVs) as measured by qPCR and normalised relative to RNU6-2. Data pooled from 3 individual experiments that were performed in triplicates. Statistical significance was determined using a two-tailed Student's t-test where $*p < 0.05$ and $**p < 0.01$. (**C**) Bar graphs (mean+SEM) showing the relative quantity of miR-142-3p as measured by qPCR and normalised relative to RNU6-2 in FACs sorted $CD11c^+CD4^-$BM-DCs cultured alone (DC alone) or with dTregs (DC co-culture) overnight at a 1:1 ratio. Data pooled from 3 individual experiments that were performed in triplicates. Statistical significance was determined using a two-tailed paired Student's t-test where $*p < 0.05$.

and BM-DCs in our initial screen. Given these results, we performed a qPCR analysis on RNA extracted from BM-DCs co-cultured with Tregs to assess whether this miRNA was indeed transferred to DCs. We observed a small but significant increase (p = 0.0267) in the presence of this miRNA in the DCs following interaction with dTregs (Fig. 5C). Taken together, our data suggests that Tregs package miRNAs, such as miR-150-5p and miR-142-3p, into EVs and that these may deliver their miRNA contents to DCs.

miR-150-5p and miR-142-3p presence is increased in DCs following co-culture with Treg EVs. Several miRNAs have been shown to modify cytokine production in DCs, including the two miRNAs previously described[24,34]. In order to assess whether EVs played a role in the intercellular transfer of Treg derived miRNA to DCs, which might help explain the cytokine profiles shown in Fig. 2D, EVs derived from dTregs and the control $CD4^+FoxP3^-$ cells were isolated and co-cultured independently with BM-DCs for 24 hours at a ratio of EVs isolated from 100 donor cells to one recipient cell[35]. After 24 hours, BM-DCs were harvested, washed thoroughly and both miR-150-5p and miR-142-3p presence was measured by qPCR. We observed that both miR-150-5p and miR-142-3p were significantly increased in DCs co-cultured with the Treg derived EVs compared to DCs co-cultured with $CD4^+FoxP3^-$ EVs (Fig. 6A). To further confirm that this was due to the uptake of EVs rather than an induction of these miRNAs in the recipient cell, we utilised Dicer deficient mice as BM-DCs isolated from these mice lack mature miRNAs[19]. Addition of Treg derived EVs, but not EVs isolated from a $CD4^+Foxp3^-$ cell line, to $Dicer^{-/-}$ DCs resulted in the significant increase of miR-150-5p in these cells (Fig. 6B). Although an increase in miR-142-3p within the Dicer deficient DCs was observed this was not significant (Fig. 6B). Overall these observations suggest that miRNAs present in Treg EVs are acquired by BM-DCs and may modulate DC function by affecting the production of cytokines.

Discussion

The data presented in this manuscript suggests that regulatory T cell derived EVs can modify DCs with respect to the cytokines release following TLR activation. Taken together, the observations that these DCs produce less pro-inflammatory cytokine IL-6 in favour of cytokines such as IL-10 suggest that Treg derived EVs may induce a

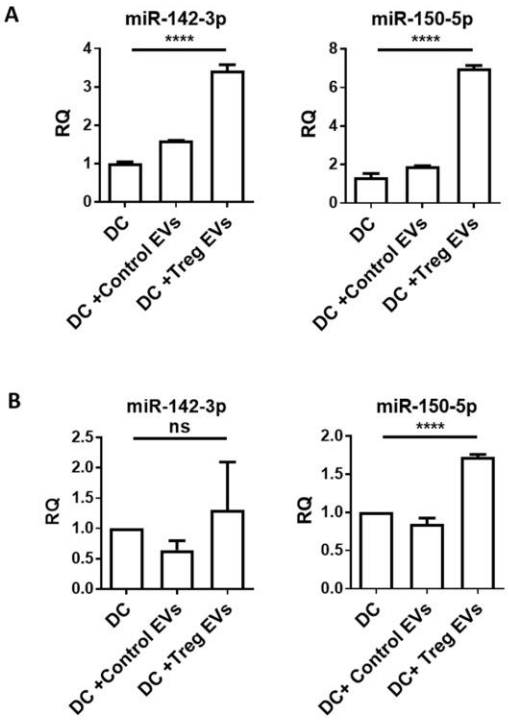

Figure 6. Treg EV derived miRNAs are transferred into DCs. (**A**) Bar graphs show relative quantity (RQ) of miR-142-3p and miR-150-5p in untreated B6 derived DCs (DC), control T cell EV-treated DCs (DC+Control EVs), or Treg EVs-treated DCs (DC+Treg EVs). DCs were treated with EVs for 24 hours, washed thoroughly and miRNA presence was measured by qPCR normalised to RNU6-2. Data represents mean+SEM pooled from 3 individual experiments that were performed in triplicates. Statistical significance was determined using One-way ANOVA and Tukey's multiple comparison test, ****$p < 0.0001$. (**B**) Bar graphs show RQ of miR-142-3p and miR-150-5p in untreated Dicer deficient-derived DCs (DC), control T cell EVs-treated Dicer-deficient DCs (DC+Control EVs) or Treg EVs-treated Dicer-deficient DCs (DC+Treg EVs). Dicer-deficient DCs were treated with EVs for 24 hours, washed thoroughly and miRNA presence was measured by qPCR and normalised to RNU6-2. Data shown is mean+SEM representative of 2 experiments that were performed in technical triplicates. Statistical significance was determined using One-way ANOVA and Tukey's multiple comparison test, ****$p < 0.0001$ and ns = not significant.

'tolerogenic' phenotype in DCs. Given that miRNAs play an important role in modulating DC cytokine profiles[24], we assessed whether dTregs can modulate DC via the intercellular transfer of small RNAs, miRNAs. Human $CD4^+$ T cells have been shown to transfer EVs containing miRNA to APCs during immune recognition leading to immune modulation[22]. In agreement with this study, we demonstrated that miRNAs present in murine Treg derived EVs, are acquired by DCs. Indeed, we hypothesise that this intercellular transfer of miRNAs may result in the modification of the cytokine profile seen in treated DCs. Interestingly EV exposure/transfer of miRNAs did not modulate the expression of the co-stimulatory molecule CD80 which was observed following exposure to Treg cells. However many studies have found that over expression of a specific miRNA in BM-DCs did not affect cell surface markers expression while the cytokine profile was affected, so our findings are in line with published data[24]. The functional significance of this is at present unknown. However, we suggest that modification of DCs by EVs will complement/add to the other pathways utilised by Treg cells to induce a 'tolerant' APC. However given that several studies have used rat and mouse Treg derived EVs to induce tolerance in the setting of transplantation it is possible that the EVs is one of the major pathways by which Tregs function[36] through modification of cytokines released by DCs by transferring miRNAs.

Inhibiting pro-inflammatory cytokines production in DCs has been linked to several miRNAs[24]. These include, and are not limited to, miR-21 which inhibits IL-12p35 production, and miR-148/152 which suppress both IL-6 and IL-12 production. miR-21 was significantly expressed in the Tregs used in this study compared to BM-DCs, and this miRNA, as well as miR-148 and miR-152, were identified as present at significant high levels in our Treg EV screen. We however chose to focus on two miRNAs with cytokine modulating properties that

were identified as being highly expressed in Treg rather T cell derived EVs; miR-142-3p and miR-150-5p. Ectopic expression of miR-142-3p in human DCs leads to decreased production of IL-12, IL-6 and TNFα after TLR ligation[34,37]. In addition, Sun *et al.* showed that the 3′UTR on IL-6 was a target of miR-142-3p[38]. Transfecting splenic $CD11c^+$ murine DCs with pre-miR-142-3p resulted in reduced endogenous levels of IL-6. Conversely knocking down miR-142-3p by transfecting DCs with a miR-142-3p "locked nucleic acid" (LNA) probe led to increased IL-6 production following LPS activation. In these experiments the authors also observed that this miRNA did not modify IL-10 levels following TLR activation[38].

Recently miR-150-5p was shown in human $CD4^+$ T cells to regulate IL-10. Inhibiting miR-150 in primary T cells was correlated with reduced IL-10 production following activation with anti-CD3 and CD46 antibodies[39]. Given these findings we suggest that transferring both miR-150-5p and miR-142-3p may be linked to the increased IL-10 and decreased IL-6 productions seen in DCs treated with Treg derived EVs, respectively. Whether acquisition of other miRNAs, such as miR-21, also present in the Treg derived EVs resulted in production of IL-10 following TLR ligation requires further studies. miR-21 has been shown to indirectly affect IL-10 production. Transfection of pro-miR-21 into RAW264.7 cells led to increased IL-10 production following LPS activation. The authors of this data concluded that LPS induced miR-21 targets PDCA4, a negative regulator of IL-10, leading to increased IL-10 production[40].

In addition to modulating cytokine production, miR-142-3p has also been shown to attenuate phagocytosis. Overexpression of a miR-142-3p mimic in primary human monocytes and DCs reduced *E coli* fluorescent OVA as well as antibody-mediated phagocytosis, compared to controls[37,41]. Overexpression of miR-142-3p in human DCs has been shown to affect at least 40 genes, some of which are associated with phagocytosis as well as cell signalling associated genes such as PKCα and cell mobility such as N-Wasp[37]. Overexpression of miR-142-3p in dendritic cells has also been linked with decreased T cell activation, which was independent of any changes to MHC or co-receptor expression on the DCs[41]. Overexpression of this miRNA did not affect MHC class II, CD80 or CD86 expression compared to control, non-manipulated, DCs[41]. Given these findings and the observation that miR-142-3p is increased in DCs following exposure to both Tregs and their EVs we suggest that miRNA transfer by Tregs may play a key functional role in their suppressive function with respect to APCs function.

Our flow cytometry observations suggests that Treg derived EVs are acquired by DCs. However, the possibility exists that the EVs do not enter the cell and instead adhere to the surface of the DCs. Several studies have shown that miRNAs present in T cell and Treg derived EVs directly affect validated target molecules present within the recipient cell. For example, c-Myb mRNA, an identified target for miR-150-5p, was downregulated in a miR-150-5p negative hepatocyte derived cellular carcinoma cell line following exposure to human Treg derived EVs containing this miRNA[33]. It is therefore feasible that the miRNAs present in our EVs will affect their mRNA target.

EVs have been shown to contain many other types of RNA species in addition to miRNAs some of which may modify cytokine expression. MiRNAs constitutes only a small fraction of small RNAs found in immune and non-immune cell derived EVs and other species have been identified including, snoRNA, piRNA, lincRNA, rRNA, tRNA[42] and Y RNA[43]. Indeed snoRNA202 was observed in our dTreg derived EVs. Recently, Y RNA, identified in cardiosphere-derived cell exosomes, was linked to IL-10 expression and secretion[43]. Transfection of macrophages with this Y RNA lead to increased production of this cytokine. Whether other RNA species are present in Treg EVs is out with the scope of this manuscript however given the changes to cytokine expression seen in DCs following treatment with these EVs it is possible that the changes seen are due to the transfer of a combination of RNA species or indeed a combination of miRNAs and cells surface proteins.

We suggest that the intercellular transfer of miRNAs to DCs via EVs is a novel mechanism by which Tregs modify DCs and may represent an important pathway to prevent autoimmunity. Transfer of several miRNA with the potential to induce non-inflammatory cytokine release by DCs may help create a 'tolerogenic' environment, although whether the same occurs *in vivo* and how this is modulated in autoimmune conditions has yet to be elucidated. Our findings could provide valuable information regarding the modification of Tregs for therapeutic use. Given that Tregs are now in the clinic[44], creating Tregs that deliver miRNAs to DCs via EVs may help increase the efficacy of this therapy in patients receiving a transplant or with autoimmune diseases.

Methods

Ethics Statement. These studies were approved and conducted in accredited facilities in accordance with The Home Office UK Animals (Scientific Procedures) Act 1986 (Home Office license number PPL 70/7302).

Mice. Female BALB/c and C57BL/6 (B6) mice were purchased from Harlan UK Ltd. (Biscester, Oxford, UK) and maintained under sterile conditions (Biological Services Unit, New Hunt's House, King's College London). Rosa26-ERT2-Cre Dicer floxed/floxed (Dicer knockout mice) were a kind gift from Dr Mark Wilson (Genentech, South San Francisco, California, USA). The Home Office (UK) approved all mouse protocols utilized in this study.

Flow cytometry. All antibodies were purchased from Thermo Fisher Scientific (Paisley, UK) unless otherwise stated and cells were stained using FACS buffer (PBS containing 5 mM EDTA and 1% FCS). Cells were stained with antibodies specific for the following markers: CD4 (GK1.5), FoxP3 (FJK-16s), CTLA-4 (UC10-4B9), CD73 (eBioTY/11.8), CD11c (N418), $H2K^d$ (SF-1-1.1.1), CD80 (16-10A1), CD86 (GL-1), $I\text{-}A^d$ (39-10-8), CD40 (3/23) (BioLegend) and CD25 (PC61) (BD Biosciences). For intracellular staining, cells were fixed and permeabilised with Foxp3/Transcription Factor Staining Buffer according to manufacturer's protocols (Thermo Fisher Scientific). Data was acquired on either a BD FACSCalibur™ or BD LSRFortessa™ (BD Biosciences, Franklin Lakes, New Jersey, USA) and analysed using FlowJo 10 software (Tree Star, Ashland, Oregon, USA).

Bone marrow-derived DC (BM-DC) isolation and culture. Red blood cell-depleted BM cells were incubated with an antibody mixture of YTS-191 (rat anti-mouse CD4), YTS-169 (rat anti-mouse CD8), M5-114 (rat anti-mouse MHC class II) and RA3-3A1 (rat anti-mouse B220) for 30 minutes at 4 °C. Washed cells were then incubated with polyclonal sheep anti-rat IgG coated Dynabeads® (Thermo Fisher Scientific) and antibody labelled cells were removed using a magnet. DC progenitors were cultured in complete media (RPMI-1640, 10% heat-inactivated FCS (General Electric (GE) Healthcare Life Sciences, Buckinghamshire, UK), 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES and 50 μM β2-mercaptoethanol (all from Thermo Fisher Scientific) supplemented with GM-CSF at 37 °C/5%CO_2 for 7 days. Media containing fresh GM-CSF was added on days 2 and 4. At the end of the culture >97% of the cells expressed CD11c (Supplemental Fig. 1). To isolate DCs from Rosa26-ERT2-Cre Dicer floxed/floxed BM an alternative protocol was utilised. Red blood-cell depleted BM cells were cultured in RPMI-1640 with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2mM L-glutamine supplemented with 200 ng/ml recombinant mouse GM-CSF (Peprotech) at 37 °C in 5% CO2. On days 2, 5 and 7, the media was changed with fresh media (above formulation) with a final concentration of 200 nM of 4-hydrotamoxifen (4-OHT). On day 10, DCs were harvested for experiments and gave a purity of >80%.

Treg culture, re-stimulation and suppression assay. Experiments were performed using a previously established BALB/c specific Treg line (dTregs)[26]. $CD4^+CD25^+$ T cells were isolated from B6 mice and stimulated once per week by co-culture with irradiated (30 Gy) allogeneic BALB/c DCs at a 1:4 (DC:Treg) ratio in complete media. IL-2 (10 units/ml) was supplemented on days 1, 2 and 4, post re-stimulation. For suppression assays, responder $CD4^+$ T cells were isolated from B6 splenocytes using the 'Dynal® Mouse $CD4^+$ Negative Isolation Kit' (Thermo Fisher Scientific) according to manufacturer's protocols. APCs were enriched from BALB/c splenocytes using a negative bead selection protocol with YTS-169, YTS-191 and Dynal® beads. Co-cultures contained 2.5×10^4 dTregs, 5×10^4 APCs and 2.5×10^4 $CD4^+$ T cells in complete media and were maintained at 37 °C for 72 hours, where 1 μCi ^{3}H-thymidine was added in the last 18 hours of culture. Cell proliferation was measured by beta-plate liquid scintillation counting (LKB Wallac 1205 Betaplate® liquid scintillation counter, Austria).

EV isolation and identification. dTregs maintained in EV-depleted-FCS complete media were incubated overnight in flasks coated with anti-CD3 and anti-CD28 antibodies (5 μg/ml). Culture supernatants were then depleted of cells via centrifugation at 581 × g before being passed through a 0.22 μm filter (Merck Millipore, Billerica, Massachusetts, USA). Small EVs (up to 220 nanometers in size) were isolated from the supernatants using one of two EV isolation methods: ultracentrifugation[20] and ExoQuick-TC™ (Systems Biosciences, Palo Alto, California, USA) depending on the downstream application. For electron microscopy (EM) visualisation and uptake studies ultracentrifugation was performed whilst for miRNA studies ExoQuick-TC was used. EV isolation via ultracentrifugation purification was performed using a Sorvall Discovery 100 centrifuge with a Sorvall T890 rotor, EVs were pelleted at 100,000 × g at 4 °C for 90 mins, washed with PBS then a further 90 mins spin was performed to re-pellet EVs. ExoQuick-TC™ solution was used according to the manufacturer's protocol. EVs were suspended in PBS. For identification, EVs were fixed with 4% paraformaldehyde, loaded onto carbon-coated Formwar EM grids for 20 mins. Grids were washed twice with PBS, fixed with 1% glutaraldehyde for 5 minutes. Grids were washed then stained with saturated aqueous uranyl for 10 minutes before analysis using Tecnai T12 BioTWIN electron microscope. Alternatively, a NanoSight LM10 (Nanosight, Malvern, Kk), with kind permission of Dr Sean Davidson (The Hatter Cardiovascular Institute, UK) was used to measure the concentration and size of the particles isolated. Constant flow injection was used and 5 videos of 30 seconds duration were recorded.

DC and Treg co-culture for confocal microscopy. dTregs were labelled with 1 μM 5(6)-CFDA-SE (Carboxyfluorescein Diacetate, Succinimidyl Ester, CFSE) mixed isomers (ThermoFisher Scientific) and co-cultured with BALB/c DCs at a 1:1 ratio. Real time imaging was performed using a Nikon Eclipse C1 microscope over 1300 seconds at 37 °C.

DC and Treg co-cultures for miRNA transfer analysis. BALB/c BM-DCs were co-cultured with dTregs at a 1:1 ratio for 18–24 hours. Total cells were harvested, stained with fluorescently conjugated antibodies specific for CD4 and CD11c and $CD11c^+CD4^-$ DCs were isolated by FACS sorting (FACS Aria II, BD). Control DCs cultured in the absence of dTregs were also sorted to nullify the influence of this process on the miRNome of the DCs. Sorted DCs were resuspended in TriZOL (Thermo Fisher Scientific) and stored at −80 °C in preparation for total RNA extraction.

BM-DC and EV co-culture. 4×10^7 dTregs were labelled with 1 μM 5(6)-CFDA-SE (Carboxyfluorescein Diacetate, Succinimidyl Ester, CFSE) mixed isomers (ThermoFisher Scientific) and activated as \described. EVs were isolated using ultracentrifugation and added to 4×10^5 BALB/c derived BM-DCs (day 6) and after 24 hours of co-culture cells were assessed by flow cytometry. To assess cytokine production, Treg EV treated BM-DCs were treated with 100 ng/mL lipopolysaccharides (LPS, Sigma-Aldrich, Germany) for 24 hours and culture supernatants were collected for cytokine analysis. To assess miRNAs, Treg EV treated BM-DCs were harvested, washed twice with PBS and lysed with TRIzol® (Thermo Fisher Scientific) for RNA extraction. Controls were EV untreated DCs.

RNA, extraction quantification and qPCR. Total RNA was extracted from DCs and Tregs using a phenol/chloroform approach combined with silica-membrane-based RNeasy spin columns (Qiagen, Hilden, Germany). Cells were lysed using TRIzol® and mixed with chloroform. The upper aqueous layer was collected and RNA was isolated using RNeasy spin columns, as per manufacturer's protocols. Contaminating DNA was removed by treating the eluted RNA with the 'TURBO DNase Kit' (Thermo Fisher Scientific) according

to manufacturer' protocols, after which the solution was mixed with phenol-chloroform and the aforementioned process repeated to obtain a pure RNA sample. Total RNA was sent to Exiqon for microarray analysis. Alternatively, complementary DNA (cDNA) was synthesised for qPCR analysis, as detailed below. EVs miRNA was extracted using a SeraMir kit (Systems Biosystems) following the manufacturer's protocols. Total RNA profile was assessed using an Agilent 2100 Bioanalyser and total RNA nano chips and quantified by NanoDrop™ spectrophotometer (Thermo Fisher Scientific).

cDNA fragments were synthesised using the 'miScript RT Kit' (Qiagen, Hilden, Germany), according to manufacturer's protocols. qPCR reactions were set up with the miScript SYBR Green PCR Kit and primers specific for miR-125b-5p, miR-29a-3p, miR-182-5p, miR-142-3p, miR-150-5p, miR-384-5p, SCARNA17 and RNU6-2, as per manufacturer's protocols and run on the Applied Biosystems™ ViiA™ 7 Real time PCR system. Data was analysed using the $\Delta\Delta C_T$ method where SCARNA17 and RNU6-2 were used as housekeeping controls. The EV miRNA screen was normalised using the global normalisation method with RNU6-2 as an endogenous control.

EV-packaged miRNAs were retrotranscribed and preamplified as previously reported[45] and were profiled by quantitative polymerase chain reaction (qPCR) on a QuantStudioTM 12 K Flex Real-Time PCR System (Life Technologies, Thermo Fisher Scientific, USA), a platform that simultaneously measures 754 miRNAs (miRBase v14). Global mean was selected as the best normalization method. MiRNA expression was determined using the relative quantification 2-ΔCrt[46].

Cytokine measurements. Culture supernatants were analysed using BD™ Cytometric Bead Array Flex Set according to the manufacturer's protocols to test for mouse TNF, IL-6 and IL-10 cytokines. Beads were acquired on BD LSRFortessa™ and analysed using FCAP Array v3.0.1 software. IL-12 was analysed using an IL-12 ELISA as previously described[47].

Statistical analysis. Data represents mean ± standard deviation. Statistical significance was determined using student's t-tests and one-way ANOVAs with Tukey's multiple comparisons test. Data was analysed using PRISM software (GraphPad Software, Inc, California, USA).

Data Availability Statement. The datasets generated during and/or analysed in the current study are available from the corresponding author on reasonable request.

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Author Contributions

L.A.S. and G.L. designed the study. S.T., D.B., M.S., Q.P., M.L., N.C., L.D., L.M.C. and L.A.S. performed experiments presented in the manuscript. L.A.S., S.T., D.B., G.L. and R.L. were responsible for interpretation of the data. V.B. and L.D. performed miRNA profiling. S.T., M.L. and N.C. and L.A.S. performed all the exosome experiments. D.B. performed the Treg miRNA and cell sorting flow cytometry experiments. L.M.C. performed the confocal imaging experiments. L.A.S. wrote the paper.

Additional Information

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Transplant International

REVIEW

Treg therapy in transplantation: a general overview

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SUMMARY

Solid organ transplantation remains the treatment of choice for end-stage organ failure. Whilst the short-term outcomes post-transplant have improved in the last decades, chronic rejection and immunosuppressant side effects remain an ongoing concern. Hematopoietic stem cell transplantation is a well-established procedure for the treatment of patients with haematological disorders. However, donor T cells are continually primed and activated to react against the host causing graft-versus-host disease (GvHD) that leads to tissue damages and death. Regulatory T cells (Tregs) play an essential role in maintaining tolerance to self-antigens, preventing excessive immune responses and abrogating autoimmunity. Due to their suppressive properties, Tregs have been extensively studied for their use as a cellular therapy aiming to treat GvHD and limit immune responses responsible for graft rejection. Several clinical trials have been conducted or are currently ongoing to investigate safety and feasibility of Treg-based therapy. This review summarizes the general understanding of Treg biology and presents the methods used to isolate and expand Tregs. Furthermore, we describe data from the first clinical trials using Tregs, explaining the limitations and future application of these cells.

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Introduction

Solid organ transplantation is the treatment of choice for many end-stage organ failure [1], resulting in marked improvements in both morbidity and mortality. As a result of improved surgical technique, closer co-ordination between transplant centres and better immunosuppression, short-term results are excellent. Despite patient survival rates greater than 90% 1 year after surgery, long-term acceptance still remains a challenge due to chronic rejection and the toxicity of the immunosuppressive drugs causing infections, organ failure and cancer [2–4]. 'Operational tolerance' (OT) [5] remains the ultimate goal whereby patients achieve stable graft function without immunosuppression in an immunocompetent host. Whilst achieving a state of OT is rare, in the case of liver transplantation approximately 20% of recipients have been successfully weaned off immunosuppression [6] and this percentage increased with time from the transplant [7]. In reality, studies evaluating OT have been conducted on a selected group of patients whilst the large majority are maintained on immunosuppression lifelong. Due to ongoing concerns regarding immunosuppressant toxicity and chronic rejection, there is greater impetus to identify alternative immunosuppressant strategies.

Hematopoietic stem cell transplantation (HSCT) is an established procedure concerning the infusion of

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autologous, syngeneic or allogeneic stem cells for several high-risk hematologic malignancies. The success of allogeneic HSCT depends on a multitude of parameters [8] type and stage of the underlying disease, age of the patients, human leucocyte antigen (HLA) disparity between donor/host and intensity of the pretransplantation conditioning regimen [9,10]. The main side effect of HSCT is graft-versus-host disease (GvHD) where donor T cells recognize the host minor and major histocompatibility antigens and proliferate, damaging target tissues [11] However, donor T cells are key for graft-versus-leukaemia (GvL) effect as well, and their depletion although abrogates GvHD abolishes the GvL effect. Patients undergoing GvHD receive an immunosuppressive regimen [12] responsible of many side effects but necessary to limit T-cell activation. GvHD can occur in acute and chronic forms according to time from transplantation and the type of response [13]. Although the post-transplant outcomes depend on the initial disease status, only 50–80% of patients with acute GvHD [14] and 40–50% of patients with chronic GvHD respond to steroidal therapy [15]. As a result, there is a need for alternative and more effective strategies to modulate the ongoing immune response.

One identified approach involves the use of regulatory T cells (Tregs) as a cellular therapy for the treatment of GvHD [16] and for limiting immune responses to allograft after solid organ transplantation [17].

In 1995, Sakaguchi *et al.* [18] identified for the first time a small population of $CD4^+$ cells that expressed high levels of IL-2 α-chain receptor (CD25), whose depletion resulted in autoimmune diseases whilst their transfer to neonatally day 3 thymectomized mice prevented the disease. These cells called Tregs have a pivotal role in maintaining peripheral immunological tolerance, by preventing autoimmunity and chronic inflammation. In 2003, the transcriptional regulator forkhead box P3 (FoxP3) was discovered as a master control gene for mouse Tregs [19,20]. In recent years, preclinical studies have demonstrated how adoptive transfer of Tregs inhibited GvHD [21–23] and prevented/delayed allograft rejection [24,25]. In solid organ transplantation, we and others have demonstrated that graft-specific Tregs displayed greater potency against graft rejection than polyclonal Tregs [26–28]. Together, these data supported the use of Tregs in the clinic and in 2009, the first trial using Tregs was published, opening a new field of investigation [29]. Herein, we provide an overview of human Treg heterogeneity/function and focus on the strategies used to isolate, expand and infuse Tregs under good manufacturing practice (GMP) conditions. Finally, we describe data from published papers and ongoing clinical trials using Tregs as cellular therapy, highlighting the limitations and future applicability of these cells within the transplant field.

Tregs: general overview

Heterogeneity of Tregs

The multiple subpopulations of Tregs are distinguished by the expression of different cell surface markers, mechanisms of activation and how they function (reviewed by us in [30]). One of these subpopulations is the $CD4^-CD8^+$ Tregs which can suppress target cells using a range of different mechanisms including the release of immunosuppressive cytokines and the induction of target cell death. However, despite the increasing progress to understand these cell types and their potential in solid organ transplantation [31], they are not currently available for clinical use. Thus, we will focus mostly on the best characterized Tregs which are the thymus-derived $CD4^+$ Tregs (tTregs) which constitutively express CD25 and FoxP3 and represents 5–10% of all peripheral $CD4^+$ T cells [32]. Whilst in the mouse the expression of neuropilin-1 has helped in distinguishing between tTregs and peripheral-derived Tregs (pTregs) [33,34] in human, this is not possible [35]. Currently, the only way to distinguish tTregs is the evaluation of the Treg-specific demethylated region (TSDR), an evolutionarily conserved noncoding element within the FoxP3 gene locus, which is fully demethylated in tTregs [36]. However, the evaluation of TSDR methylation status can only be a tool in diagnosis or clinical trial monitoring but not used for Tregs isolation. The best marker to distinguish and isolate Tregs in combination with CD4 and CD25 is the α-chain of IL-7R (CD127) [37]; its expression inversely correlates with FoxP3 and suppressive Treg function [37]. In 2009, Miyara *et al.* demonstrated that human Tregs in peripheral blood are heterogeneous and consists of three main subpopulations based on their expression levels of CD45RA and FoxP3/CD25 [38]. Tregs can be divided into (i) naïve/resting and very stable cells expressing $CD45RA^+FoxP3^{low}$; (ii) effector Tregs expressing $CD45RA^-FoxP3^{high}$; and (iii) cytokine-producing Tregs, expressing $CD45RA^-FoxP3^{low}$. Naïve Tregs are considered the 'real Tregs'; they are very suppressive and fully demethylated in the FoxP3 locus.

Among pTregs, arising from conventional $CD4^+CD25^-$ T cells (Tconv) in the periphery under specific conditions, are the Th3 and the Tr1. The

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presence of TGF-β and IL-4 promotes the induction of Th3 cells which in turn predominately secretes immunosuppressive TGF-β [39], whereas the presence of IL-10 and IFN-γ induces Tr1 cells which predominantly secretes IL-10 into the microenvironment [40,41]. Another type of pTregs are the induced $CD4^+CD25^+FoxP3^+$ pTregs which are generated from peripheral $CD4^+FoxP3^-$ T cells upon activation and in the presence of TGF-β and IL-2 [42]; these Tregs display similar cell surface markers as tTregs and function by contact-dependent mechanisms and the release of immunosuppressive cytokines. TSDR methylation status is a key to distinguishing between the thymus-derived $CD4^+CD25^+FoxP3^+$ and the peripheral-derived $CD4^+CD25^+FoxP3^+$ Tregs.

Treg suppression mechanisms

Tregs employ a plethora of contact and non-contact-dependent mechanisms to exert their suppressive function on different cells like $CD4^+$ and $CD8^+$ T cells, macrophages, dendritic cells (DCs), natural killer (NK) and B cells. Thornton and Shevach demonstrated that Tregs require TCR stimulation to suppress in an antigen nonspecific manner [43]. From a functional perspective, the various potential suppressive mechanisms could be divided into four 'modes of action': (A) metabolic interference, (B) inhibitory cytokine release, (C) cytolysis and (D) targeting antigen presenting cells (APCs) (extensively reviewed in [44]).

Briefly, (A) T cells depend on IL-2 for survival and proliferation, Tregs constitutively express high levels of CD25 which depletes IL-2 from the microenvironment and limiting its availability for T-cell functions [18]. Additionally, CD39 and CD73 are ecto-enzymes found on the surfaces of Tregs. Firstly, CD39 converts pro-inflammatory extracellular adenosine triphosphate into adenosine monophosphate (AMP); secondly, CD73 converts AMP into anti-inflammatory adenosine [45]. (B) Tregs can release immunosuppressive cytokines such as IL-10, IL-35 and TGF-β to prevent T-cell proliferation and maturation of APC [46–48]. (C) Tregs secrete granzymes and perforins which cause apoptosis of target cells [44]. (D) Tregs are the only T-cell subpopulation that constitutively expresses CTLA-4; it binds CD80/CD86, the co-stimulatory molecules expressed by APCs, to block their binding to CD28, thus limiting T-cell activation. Furthermore, CTLA-4 can also downregulate DCs' activity via trans-endocytosis or extraction of CD80 and CD86 resulting in diminished co-stimulation [49]. Very recently, a novel mechanism of Treg suppression was discovered by us and others. It refers to the release of nano-sized vesicles called exosomes that are immunomodulatory. We demonstrated that Treg-derived exosomes inhibited T-cell proliferation *in vitro* via CD73 molecules found on the surfaces of these exosomes [50]. Additionally, Okoye *et al.* [51] have shown that Treg-derived exosomes prevented autoimmune diseases *in vivo* which was attributed to the presence of inhibitory microRNA within these exosomes. Another study demonstrated that the adoptive transfer of Treg-derived exosomes into a rat model of kidney transplantation prolonged the survival of the allograft [52]. Taken together, these studies demonstrated that Tregs can suppress the immune response via different mechanisms.

Treg manufacturing for clinical use

Source of Tregs and their isolation

Most preclinical studies source their Tregs cellular product from either peripheral blood (PB) or umbilical cord blood (UCB). A pioneering study in 2006 by Hoffmann *et al.* [53] described for the first time a GMP procedure for the isolation of $CD4^+CD25^+$ T cells from standard leukapheresis product. Isolation was carried out by CliniMACS (CliniMACS TM Instruments, Miltenyi Biotec Bergisch Gladbach, Germany) a clinical-scale magnetic enrichment of cells in a closed and sterile system. This was performed in a two-stage method; firstly, depletion of $CD19^+$ cells followed by an enrichment of cells expressing CD25 molecules. This has now become a well-established procedure for GMP isolation. Di Ianni *et al.* [54] applied this isolation procedure to 72 leukapheresis products. They isolated a mean of 263×10^6 Tregs, and of these cells, 79.8 ± 22.2% were $FoxP3^+$. Recently, our group published the first reports of the manufacture of clinical-grade Tregs from prospective liver and renal transplant recipients [55,56]. As an example, from 150 ml of PB derived from patients with liver cirrhosis, we were able to isolate $7.14 \times 10^6 \pm 0.938$ cells with high purity.

Umbilical cord blood has been used as an alternative source for the generation of Tregs for clinical use. Brunstein *et al.* [57] isolated a mean of 6.6×10^6 cells from one UCB with a mean purity of 66%.

Although the CliniMACS has been extensively used to isolate Tregs under GMP conditions [58,59], the purity of the cells obtained is not optimal as they are contaminated with $CD25^{low}$ Tconv. This limitation has hampered the generation of antigen-specific Tregs

production for which high purity of Tregs is needed. An alternative method for Tregs isolation is the flow cytometry-based purification. This offers the advantage of a highly pure cell product isolated using a combination of multiple surface markers (e.g. CD25 and CD127). Unfortunately, it presents considerable regulatory challenges in the EU (Directive 2003/94/EC and its Annex 2) and to date, only one group in Europe and two in the USA have obtained regulatory approval to use flow-sorted Tregs and published their clinical strategy (University of Minnesota, USA [57]; University of California, USA [60] and University of Gdansk, Poland [29]).

Treg expansion

Considering the low number of Tregs present in both PB and UCB, the infusion of a large number of freshly isolated Tregs is difficult to achieve [61]. In the setting of HSCT, Tregs are isolated from the donor and a larger number of cells can be obtained. However to increase the number of cells for infusion, both in GvHD and solid organ transplantation, Tregs have been expanded *ex vivo* using anti-CD3/CD28-coated beads in the presence of high dose of IL-2 (polyclonal expansion). One caveat of Treg isolation using immunomagnetic technique is that the resultant cells are contaminated with effector T cells. To avoid the infusion of activated effector T cells, we and others have developed Treg expansion protocols using drugs like rapamycin or *all-trans* retinoic acid (ATRA) [62,63]. The positive effect of rapamycin on Tregs viability and expansion has been observed firstly *in vivo*. Kidney-transplanted patients receiving a rapamycin-based immunosuppression regimen presented an increased proportion of Tregs as compared to patients on calcineurin inhibitors [64]. *In vitro*, rapamycin significantly reduces the undesired expansion of effector T cells allowing proliferation of Tregs that are independent from mTOR pathway for their cell cycle progression [65]. In addition, rapamycin confers to the expanded Tregs higher stability and suppressive capacity [66] as showed by us *in vitro* and in GvHD mouse models [63].

The alternative drug ATRA affects T-cell fate by contributing to Treg differentiation in combination with TGF-β [67,68]. Although its role in Treg induction is well established, the effects on tTregs are still controversial and for this reason, no GMP expansion protocol has been developed yet.

After cell-sorting isolation, antigen-specific Tregs can be generated and expanded *ex vivo* under GMP conditions. We in collaboration with Tang's group have recently published a preclinical protocol for the generation and expansion of antigen-specific Tregs [69]. Tregs were cultured with previously activated (by CD40 ligand) allogeneic B cells in the presence of IL-2. These cells were more potent in suppressing alloimmune responses *in vitro* and *in vivo*, using a humanized mouse skin transplant model, when compared to polyclonally expanded Tregs.

Clinical trials using Tregs

At the end of October 2016, only few results from clinical trials have been published showing safety and feasibility of Treg infusion. However, there are several ongoing phase I/II clinical trials with Tregs in solid organ transplantation and HSCT (Table 1).

The first paper reporting the infusion of *in vitro*-expanded Tregs was published in 2009 [29]. The authors described a procedure and first-in-man clinical effects of adoptive transfer of *ex vivo*-expanded $CD4^+CD25^+CD127^-$ cells for the treatment of two patients affected by acute and chronic GvHD, respectively. Due to the restricted patient number and the procedure to isolate and expand Tregs, no conclusion about safety was drawn.

In 2011, Brunstein *et al.* [57] published results from the first phase I clinical trial using expanded Tregs from third-party UCB. The study aimed to evaluate the safety and feasibility of UCB Tregs in 23 patients with acute GvHD. Patients received a dose escalation of Tregs from 0.1 to 30×10^5 UCB Tregs/kg. No toxicities were observed after infusion, and Tregs were detected for 14 days. Although this was only a phase I clinical trial, the authors affirmed that, compared with identically treated 108 historical controls, there was a reduced incidence of grade II–IV aGvHD with no deleterious effect on risks of infection, relapse or early mortality.

In 2014, Martelli's group [70] published another study in which freshly isolated donor-derived Tregs were injected before HSCT to avoid the extensive *ex vivo* T-cell depletion of the graft. Between September 2008 and December 2012, they infused 43 patients with high-risk acute leukaemia. This study demonstrated for the first time that adoptive immunotherapy with Tregs protected from GvHD mediated by the infusion of high number of donor Tconv in patients undergoing full-HLA haploidentical transplantation. The surprising finding was the absence of GvHD in patients who received up to 10^6 Tcons/kg after an infusion of 2×10^6 Tregs. Furthermore, the immunological reconstitution was stronger and faster than the historical

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Table 1. Ongoing clinical trials with Tregs in transplantation

Study ID	Phase	Product	Indication	Status
HSCT				
NCT01903473	II	Fresh tTregs	Steroid-refractory cGvHD	Recruiting
NCT01911039	I	Fresh tTregs	Steroid-dependent/Refractory cGvHD	Recruiting
NCT00602693	I	Umbilical Cord Blood Tregs	GvHD Prevention	Ongoing but not recruiting
NCT02749084	I	Fresh tTregs	Severe Refractory cGvHD	Recruiting
NCT02526329	I	Fresh tTregs	aGvHD	Ongoing but not recruiting
NCT01937468	I	Fresh tTregs	Steroid-Refractory cGvHD	Recruiting
NCT01660607	I/II	Fresh tTregs	GvHD Prevention	Recruiting
NCT02385019	I/II	Fresh tTregs	Steroid-Refractory cGvHD	Recruiting
NCT01634217	I	Induced Tregs	Nonmyeloablative HLA Identical Sibling Donor	Recruiting
NCT01795573	I	Donor–alloantigen-reactive Tregs	aGvHD Prevention	Recruiting
SOT				
NCT02145325		Polyclonally Expanded tTregs	Living Donor Kidney transplant	Ongoing but not recruiting
NCT02129881	I/II	Polyclonally Expanded tTregs	Living donor kidney transplant	Recruiting
NCT02371434	I/II	Polyclonally Expanded tTregs	Living donor kidney transplant	Recruiting
NCT02244801	I/II	Donor–alloantigen-reactive tTregs	Living donor kidney transplant	Recruiting
NCT02091232	I/II	Belatacept-conditioned tTregs	Living donor kidney transplant	Recruiting
NCT02166177	I	Polyclonally Expanded tTregs	Liver transplant	Recruiting
NCT02188719	I	Donor–alloantigen-Reactive Tregs	Liver transplant	Recruiting
NCT02088931	I	Polyclonally Expanded tTregs	Living donor kidney transplant	Recruiting
NCT02474199	I	Donor–alloantigen-Reactive Tregs	CNI reduction in liver transplant	Not yet recruiting
NCT02711826	I	Donor–alloantigen-Reactive Tregs	Subclinical Inflammation in Kidney Transplantation	Recruiting
NCT01624077	I	Induced Tregs	Liver transplant	Ongoing but not recruiting

HSCT, haematopoietic stem cell transplantation; SOT, solid organ transplantation; aGvHD, acute Graft-versus-Host Disease; cGvHD, chronic Graft-versus-Host Disease; CNI, calcineurin inhibitor.

controls and after a median follow up of 45 months, the leukaemia relapse in patients receiving Tregs was markedly reduced. In our opinion, this could be considered a proof that Tregs do not target GvL; however, this data need to be confirmed by other studies.

More recently, a clinical trial evaluating the adoptive transfer of allogeneic Tregs into patients with chronic GvHD has been published [71]. All the five patients selected for this trial were unresponsive to the standard therapy. To our knowledge, this is the first trial adopting a combined therapy using Tregs and low dose of IL-2. All the patients tolerated the Treg products combined with an increase of circulating Tregs and disease improvement or stability. Of note, the three patients receiving IL-2 showed an increased T-cell activation; however, the clinical improvement suggests that the beneficial effects of low-dose IL-2 on Treg functions was able to control the possible expansion of effector subsets.

In another published clinical trial ('ALT-TEN'), Tr1 were used [72]. These cells have been infused into 12 patients with high-risk/advanced stage hematologic malignancies after chemotherapy conditioning and T-cell-depleted haploidentical HSCT. Tr1 were infused when no spontaneous immune reconstitution was detectable. As highlighted by the authors, this study had multiple limitations namely that eight patients died so data were obtained from four patients only. A further problem was the percentages of Tr1 in the infused cell product. In fact, the infusion of 3×10^5 $CD3^+$ cells/kg

provoked GvHD grade III–IV in one patient, suggesting that the ratio between effector cells and Tr1 cells was too high. In our opinion, further trials are necessary to establish the safety of this cell product and this will be performed as part of 'THE ONE STUDY' consortium who will test Tr1 cells as treatment after kidney transplant [73].

The only data regarding the use of Tregs in solid organ transplantation have been recently published from Okumura's group [74]. Between November 2010 and July 2012, they treated patients with end-stage liver failure who underwent transplantation from a living donor with a novel Treg-based cell therapy. Of note, all the patients were splenectomized. Recipient lymphocytes were enriched in regulatory cells after co-culture with irradiated donor cells in the presence of anti-CD80/CD86 antibodies for 2 weeks. The infused cell product contained a number of $CD4^+CD25^+FoxP3^+$ cells ranging from 0.43×10^6/kg to 6.37×10^6/kg. The immunosuppression weaning started after 6 months post-transplantation followed by a complete weaning at 18 months. Noteworthy, results came from 10 consecutive patients although a total of 40 patients were initially planned. Unfortunately, this trial was suspended because of acute cellular rejection during weaning in two patients with primary biliary cirrhosis and one with primary sclerosing cholangitis. Seven patients were successfully weaned off immunosuppression whilst the three recipients with rejection were stabilized using low dose of tacrolimus and mycophenolate mofetil. In conclusion, this Treg-enriched product seems to be safe and the results are promising. However, the effect of splenectomy in combination with Treg cell therapy has to be clarified and concerns remain about the presence of antigen-specific effector cells in the resultant cell product.

Between the ongoing clinical trials (Table 1), 'THE ONE STUDY' is an EU Consortium aiming to test different regulatory cell products in kidney transplantation [73]. Our group together with the group in Oxford led by Andrew Bushell and Paul Harden has just completed the infusion of expanded autologous Tregs in 12 patients. Four doses of Tregs ($1, 3, 6, 10 \times 10^6$/kg) have been infused 5 days post-transplant in the presence of immunosuppressive drugs. Our group started at the same time of 'THE ONE STUDY' another clinical trial called ThRIL (Table 1), investigating the safety of Tregs immunotherapy after liver transplantation. The clinical protocol involves ATG at time of transplantation, followed by tacrolimus with a switch to sirolimus at 2 months post-transplantation. Three Treg doses (same preparation of 'THE ONE STUDY') are being tested: 1, 4.5 and 6×10^6 cells/kg at 3 months post-transplantation. Three patients have already been treated with the lowest dose of Tregs. Lastly, a clinical trial from the University of Liegi (Table 1) is aiming to assess the safety of the combination of donor Treg infusion and rapamycin administration (a nonstandard immunosuppressor for this disease) in patients with steroid-refractory chronic GvHD. They will be firstly treated with rapamycin, and after 3–4 weeks, one infusion of Tregs will be administrated.

Future directions

As recently affirmed by KJ. Wood, the infusion of Tregs in transplantation is at the 'end of the beginning' [17]. This is because in the last two decades, Tregs have transformed from being an ideal candidate for OT induction and GvHD treatment/prevention to a population that can be isolated, expanded and infused *in vivo*. All published data so far indicate that Tregs are well tolerated even when high doses have been infused. However, this has also opened further lines of inquiry concerning: sources, isolation strategy, doses, timing of infusion, optimal immunosuppressive regimen and cell fate postinfusion.

The groups of MK. Levings and LJ. West have successfully isolated Tregs from discarded paediatric thymuses [75]. These Tregs have several advantages over their peripheral blood counterparts. The Treg yield in a single thymus exceeds the estimated Treg number in the entire circulating blood volume of an average-sized adult; moreover, Tregs could be clearly distinguished from Tconv and after expansion, they were more suppressive and stable than blood Tregs. However, this current source of Tregs is only from paediatric heart transplant patients.

Another step ahead for cell therapy using Tregs is the development of GMP-cell sorters. Using this strategy, the following subsets of Tregs can be obtained: $CD4^+CD25^+CD127^-CD45RA^+$ for the isolation of naïve cells [38]; $CD4^+CD25^+CD127^-CD39^{high}$ [76] for Tregs presenting stronger stability and function under inflammatory conditions; $CD4^+CD25^+CD127^-CD226^-TIGIT^+$ [77] for the exclusion of unstable Tregs after *in vitro* expansion. However, a combination between CliniMACS and cell sorting is needed to obtain higher yields of cells.

Another issue is timing of Treg infusion that has to be programmed considering the immunosuppressive regimen adopted (extensively reviewed for solid organ

transplantation by us in [78]), the type of patients and donors (death or living donor). The ongoing clinical trials are using new strategies combining Tregs infusion with the use of rapamycin as an immunosuppressive drug or, more recently, low dose of IL-2. These combined strategies could further prolong Treg survival and increase Treg stability *in vivo*, improving the outcome of cell therapy. Another advantage by prolonging Treg survival is to facilitate the induction of 'infectious tolerance' [79,80], namely the capacity of Tregs to transmit tolerance from one population to another.

In view of improving the outcome of Treg therapy in the future, it is important to understand the fate of the injected Tregs. Treg tracking in a noninvasive and safe way and suitable for GMP products remain undeveloped. A promising GMP-labelling protocol has been developed and tested in type 1 diabetic patients receiving polyclonally expanded Tregs [60]. During the expansion procedure, D-[6,6′-$^{2}H_{2}$]glucose has been added in culture and incorporated in the DNA of replicating Tregs. After labelling, cells maintained their phenotype and function and could be detected in circulation 1 year postinfusion. This protocol allows the study of circulating Tregs *in vivo* and their stability; however, to study Treg localization in tissue and their homing capacity, new techniques are under development.

Conclusions

Although much work is still to be performed, there is now concrete evidence to support Treg-based cell therapy in the clinical arena. Results coming from the ongoing clinical trials will give us additional information about the impact of these cells in the clinic. For this reason, we will only be able to conclude on their efficacy in a few years when longer term data will become available.

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Conflict of interest

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Regulatory T cell-derived exosomes: possible therapeutic and diagnostic tools in transplantation

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Exosomes are extracellular vesicles released by many cells of the body. These small vesicles play an important part in intercellular communication both in the local environment and systemically, facilitating in the transfer of proteins, cytokines as well as miRNA between cells. The observation that exosomes isolated from immune cells such as dendritic cells (DCs) modulate the immune response has paved the way for these structures to be considered as potential immunotherapeutic reagents. Indeed, clinical trials using DC derived exosomes to facilitate immune responses to specific cancer antigens are now underway. Exosomes can also have a negative effect on the immune response and exosomes isolated from regulatory T cells (Tregs) and other subsets of T cells have been shown to have immune suppressive capacities. Here, we review what is currently known about Treg derived exosomes and their contribution to immune regulation, as well as highlighting their possible therapeutic potential for preventing graft rejection, and use as diagnostic tools to assess transplant outcome.

Keywords: regulatory T cells, exosomes and immune modulation

TREG EXOSOMES – IMMUNE MODULATORS

Exosomes are small, cup-shaped, secreted membrane vesicles (approximately 50–100 nM in diameter) that are formed by the inward budding of endosomal membranes (1–6). Exosomes are released into the extracellular environment following the fusion of multivesicular endosomes with the plasma membrane (7). Several proteins involved in their biogenesis and release have been described and have recently been reviewed by Colombo et al. (7). Exosomes released by many immune and non-immune cells have been shown to have a range of physiological properties within the immune system. These include antigen presentation, immune regulation, and programed cell death, each of which is linked to the cell from which they are released (6, 7). They play an important role in intercellular communication and can act as shuttles for transferring proteins, miRNA, mRNA, and cytokines from one cell to another (8).

Many cells of the body produce these extracellular vesicles (EVs) including those of the immune system such as $CD4^+$ and $CD8^+$ T cells, B cells, and dendritic cells (DCs). Exosomes from these cells have been shown to mediate either immune stimulation (DCs) or immune modulation (T cells) (9–14). Recently, the release of exosomes by murine $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs), following TCR activation, was shown, initially by Smyth et al. (15) and later by Okoye et al. (16). In addition to $CD4^+CD25^+Foxp3^+$ cells, other murine T cells with regulatory capacities were found to also release exosomes following activation. Bryniarski et al. observed that "exosome like" particles were present in the supernatants of cultured $CD8^+$ T cells with suppressive capacity (17), whilst Xie et al. observed that $CD8^+CD25^+Foxp3^+$ T cells secreted exosomes capable of inhibiting DC induced $CD8^+$ CTL responses (18).

Exosome production by murine $CD4^+CD25^+Foxp3^+$ Tregs appears to be quantitatively greater than other murine T cells, including naïve $CD4^+$ and $CD8^+$ T cells, T helper 1 (Th1), and Th17 cells, and is regulated by changes in intracellular calcium, hypoxia, and sphingolipids ceramide synthesis, as well as in the presence of IL-2 (16). Exosomes contribute significantly to the function of murine $CD4^+CD25^+FoxP3^+$ Tregs, inhibiting the release of exosomes reversed these cells suppressive capabilities (16). In parallel, murine Tregs exosomes were found to be immune modulatory. Reduced $CD4^+$ T cell proliferation and cytokine (IL-2 and IFNγ) release was observed in their presence *in vitro* (15). The suppressive nature of Treg exosomes, in one study, has been attributed to the ectoenzyme CD73 (15). The loss of CD73 on Treg exosomes reversed their suppressive nature. Expression of both CD39 and CD73 on Tregs contributes to immune suppression through the production of the anti-inflammatory mediator adenosine (19–21). Binding of this molecule to adenosine receptors A2aR, expressed by activated T effector cells (Teffs) triggers intracellular cAMP leading to the inhibition of cytokine production, thereby limiting T cell responses (22). Given that adenosine was produced following incubation of CD73 expressing Treg exosomes with exogenous 5′AMP it is feasible that the release of exosomes expressing CD73 within the local environment increases the surface area by which this membrane-associated enzyme, and ultimately Treg suppression, can function (15).

Several molecules associated with immune modulation including CD25 and CTLA-4, were also found on $CD4^+CD25^+Foxp3^+$ Treg exosomes (15). Nolte-'t Hoen et al. have previously shown that exosomes, derived from anergic rat T cells, inhibited Teffs responses following co-culture with B cells and DCs *in vitro* (23).

These T cell-derived exosomes expressed high levels of CD25 and the authors suggested that CD25 expressing exosomes, binding to the surface of an antigen presenting cells (APC), bestows that cell with the ability to bind free IL-2 in the local environment leading to depletion of available cytokines and apoptosis of Teffs (23). Although CD25 expression was observed on Treg exosomes, this molecule may not play a role in their suppressive function given the observation that exosomes isolated from a T cell line, incapable of suppressing proliferation or cytokine production of $CD4^+$ T cells, in the presence of B cells, expressed similar levels of CD25 to Treg exosomes with regulatory function (15). A redundant role for CTLA-4 molecules has also been reported. Although present on Treg exosomes, blocking CTLA-4 did not modulate their suppressive function (15). So far, no molecules have been associated with the regulatory capacity of $CD8^+25^+FoxP3^+$ exosomes (18).

Recently, the transfer of miRNAs contained in T cell exosomes has been shown to affect the function of recipient APCs by inhibiting translation of target mRNA molecules (14, 24). Likewise, the transfer of miRNAs, including Let-7d, miR-155, and Let-7b, to Teffs through the acquisition of $CD4^+CD25^+Foxp3^+$ Treg exosomes has been shown (16). Inhibiting Let-7d expression in Treg exosomes reversed the suppressive nature of these vesicles suggesting that miRNAs present in Treg exosomes may also play a role in their suppressive capacity (16). These findings confirm those of Bryniarski et al. (17) who observed the targeted delivery of an inhibitory miRNA, miR-150, to Teffs using exosomes isolated from $CD8^+$ T cells with suppressive capacity.

Several molecules present on exosomes isolated from Teffs, DCs, and B cells have been shown to have immune modulatory properties. Whether they also contribute to the suppressive nature of Treg exosomes has yet to be validated. For example, expression of FasL on murine $CD8^+$ T cell exosomes induced death of APCs (12, 25), in addition, FasL-expressing exosomes isolated from DCs, genetically modified to express FasL, suppressed antigen-specific immune responses *in vivo* (26) and lastly, $MHCII^+FasL^+$ exosomes constitutively produced by a human B cell-derived lymphoblastoid cell lines induced apoptosis in $CD4^+$ T cells (27). Murine and human $CD4^+25^+$ Tregs express FasL (28). Whether FasL is expressed on Treg exosomes and contributes to the death of Teffs is yet to be tested. Other molecules, present on Tregs such as the inhibitory cell surface ligand programed cell death 1 ligand 1 (PDL-1) and Galectin-1 (29–31) may also be present on Treg exosomes. PDL-1 was found on mesenchymal stem cell EVs (32) and exosomes have been identified as transport vehicles for the secretion of molecules that lack a signal sequence such as Galectin-1 (33). Not only is this molecule highly expressed on Tregs it is essential for their function (34).

Regulatory T cells produce immune modulating cytokines such as IL-10, IL-35, and TGFβ (35). Presently, it is unknown whether these cytokines are contained in Treg exosomes however, expression of IL-10 and TGFβ in exosomes isolated from DCs, transduced to express these cytokines, has been shown (36, 37) as has surface TGFβ on MSC derived EVs (32). Given the aforementioned it is a theoretical possibility, that Treg exosomes may contain one or more of these cytokines.

ROLE OF Treg EXOSOMES IN TRANSPLANTATION

POSSIBLE THERAPY?

In 1990, Hall et al. observed that the adoptive transfer of $CD4^+CD25^+$ T cells resulted in long-term cardiac allograft survival in cyclosporine-treated rats (38). Since then this field of immunotherapy has been intensely studied in mouse (39–41), and recently in preclinical humanized mouse models (mice reconstituted with a human immune system and transplanted with human skin or human pancreatic islets of Langerhans) (42, 43). In the latter, human $CD4^+25^+$Tregs, expanded with anti-CD3/28 antibody coated beads, have been found to prolong islet transplant survival and function (42, 44). These positive outcomes have led to the application of humans Tregs for the prevention of graft versus host disease (GvHD) and to promote transplant tolerance (45–48). Currently, several organizations around the world are investigating the use of $CD4^+CD25^+$ Tregs to promote "tolerance" to transplanted organs. At King's College London, UK, phase I/II clinical trials are currently under way to test the safety and efficacy of using these cells in human kidney (One Study) and liver (ThRIL) transplant patients. Other clinical trials using human Tregs are also underway and are described elsewhere (49). Presently, we do not know the efficacy and efficiency of Tregs in these trials. Although Tregs are now being used in patients how they function *in vivo* is still unknown.

Given their immune modulatory capacity, the question arises, what is the contribution of Treg exosomes to transplant tolerance seen in the preclinical mouse models and can Treg exosomes be used *in vivo* as an alternative/or complementary therapy? At present, we are a long way away from using Treg exosomes in man given that the optimal Treg subset required to induce transplant tolerance is as yet unknown, as is whether they prolong graft survival in a patient setting. So why should we consider these EVs as a therapy? Several studies have suggested that inflammatory environments can subvert human $Foxp3^+$Treg cell function by converting them to Teffs *in vivo* (50, 51). However, unlike $Foxp3^+$ Tregs, adoptive transfer of human Treg exosomes are unlikely to be modified during inflammatory conditions *in vivo* (1) making them an ideal immune modulatory reagent (**Figure 1A**).

Several lines of evidence exist, some preliminary, some not, suggesting that studying these vesicles for this purpose is worthwhile, albeit challenging. So far, Yu et al. are the only group that have investigated the use of Treg exosomes as a therapy in a transplantation setting (52). These authors observed that the adoptive transfer of autologous rat Treg exosomes, post transplant, prolonged both survival and function of kidney allografts (52). Suggesting that Treg exosomes may represent an exciting new therapy for the induction of transplant tolerance.

Can this observation be translated into a human setting? Using preclinical methods to isolate and expand human Tregs, from peripheral blood of health individuals (53), we have successfully identified the release of CD63 and CD81 expressing exosomes from $CD4^+CD25^{hi}Foxp3^+$ suppressive human Tregs, following TCR activation (Agarwal et al., personal communication). Whether human Treg exosomes display molecules that can modulate the immune response *in vivo* is still being assessed. However, given that Jurkat $CD4^+$ T cells (a human T cell line) as well as human

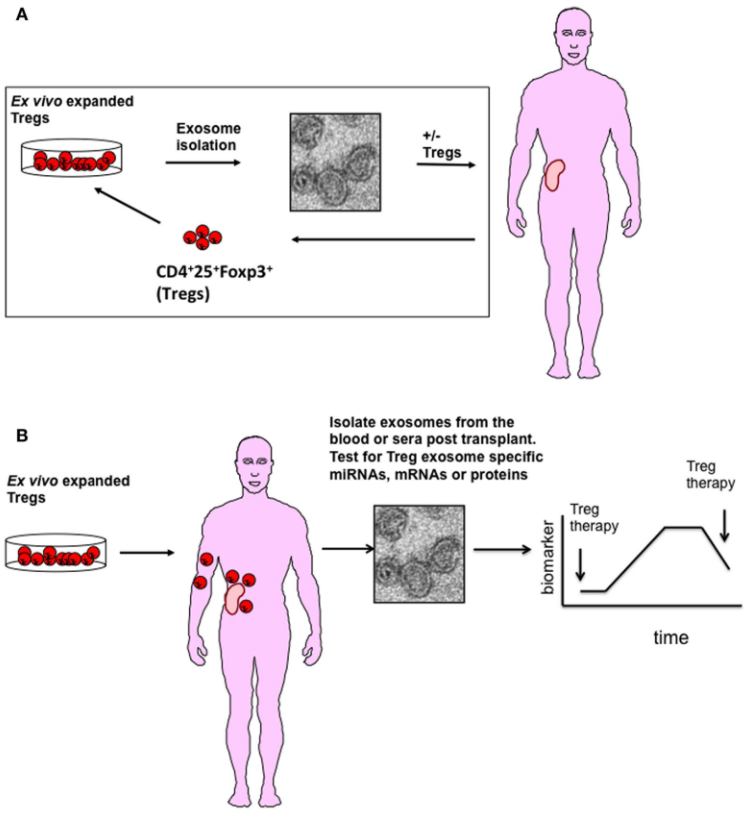

FIGURE 1 | A possible role for Treg exosome in transplantation. **(A)** Exosomes isolated from *ex vivo* expanded polyclonal or antigen-specific Tregs represent a potential adoptive therapy tool to promote transplant tolerance. Exosomes isolated from activated Tregs either alone or modified to express specific inhibitory miRNAs, chemicals, or cell surface molecules could be used in conjunction with Tregs to promote transplant tolerance. **(B)** Following transplantation, exosomes released from Tregs maybe used as a diagnostic tool to monitor activation and survival of Tregs *in vivo*. As Tregs release exosomes following activation, interaction with APCs expressing alloantigen on grafted tissue will result in exosome release. Identifying specific miRNAs expressed in Treg exosomes will help in their identification in blood or urine.

$CD3^+$ T cells, isolated from PBMCs, produce exosomes (54–56) containing molecules with potential immune regulatory effects, such as TCRs (54) and CTLA-4 (56) the possibility that human Treg exosomes contain immune regulatory molecules is very high.

Two phase I clinical trials using exosomes isolated from immature DCs have been conducted in advanced stage melanoma and MAGE-expressing non-small cell lung cancer patients (57–59). Despite a lack of antigen-specific T cell responses, stable disease was observed in some patients with tumor regression reported in one patient following treatment (60–62). These positive outcomes have paved the way for Phase II clinical trials using exosomes isolated from LPS or IFNγ activated DCs in non-small cell lung cancers. These studies have validated the efficacy and safety of exosomes as a therapy in man. In spite of these encouraging findings, several key limitations pertaining to the use of exosomes cannot be ignored. Firstly, at present there is no standardized protocol for isolating and analyzing "pure" exosomes (7). Contamination from other EVs as well as membrane free aggregates may be an issue depending on the isolation method used. Therefore, careful analyses of the purified exosomes will be required before administration. This will require the use of expensive equipment such as EM and Nanosight, which are not always readily available (7, 63). Secondly, given that exosome release by Tregs is not constitutive and requires activation using anti-CD3/CD28 antibodies, the possibility that these antibodies contaminate Treg exosome preparations is as yet untested. Additionally contaminating molecules, for example, proteins/cytokines present in media, may pose a potential problem especially as exosomes will be isolated from culture supernatants. Thirdly, the quantity of exosomes isolated and the amount required for therapy purposes are at present unknown, as is whether large-scale production of Treg exosomes is actually possible. Lamparski et al. published that 1.8–5.8 mg

of exosomes could be isolated from human monocyte derived DCs, expanded from peripheral blood leukopacks (originally containing 12–25 $\times$ 10^9 cells) higlighting the feasibility of large-scale production of DC exosomes (64). However, DCs produce these EVs vesicles constitutively making their production easier than those from Tregs, which are isolated only after activation (65). Yu et al. obtained 117 μg of exosomes from 4 $\times$ 10^9 freshly isolated rat Tregs, following activation, and the administration of 33 μg of exosomes, given over 3 time points, was sufficient to prolong the lifespan of a kidney transplant (52). Whether large quantities of pure exosomes can be isolated from human Tregs grown under GMP conditions is as yet unknown. Lastly, what happens to Treg exosomes *in vivo*, which cells acquire them and whether is it receptor driven is poorly understood. Recently, Teffs were shown to acquire Treg exosomes (16) whilst exosomes from EL4, a T cell lymphoma, have been shown to be preferential acquired by macrophages (66), perhaps via the CD169 pathway (67). Therefore, *in vivo* analysis of Treg exosomes is essential before they can be used in a clinical setting. Until all of these factors are addressed, using Treg exosomes in a transplant setting remains challenging and potential advantages remain at present theoretical.

DIAGNOSTIC TOOL?

Biomarkers are quantitatively, measurable biological parameters that help indicate health and disease. The use of exosomes as biomarkers is a relatively new concept. Although it has not yet reached clinical practice, it is one area of exosome research that is rapidly expanding, with many clinical trials focusing on their use as a diagnostic tool, particularly for cancer (**Table 1**). Several factors make exosomes suitable for this purpose, firstly, they travel through the bloodstream and can be isolated from plasma, serum, and urine (68, 69). Secondly they receive surface markers from the cell from which they are derived, such that they can be identified and isolated. Lastly, they express unique miRNA and mRNA (**Table 1**).

Valadi et al. were the first group to publish that exosomes contained RNA (8). Exosome RNA is small, typically of about 200 bases in length and lacks the 18S and 28S RNA found in cells (74). Different RNA species including small ribosomal RNA, specific tRNA fragments, long interspersed elements, and long terminal repeats, have all been found in exosomes (75). Additionally, and as discussed earlier, there is also a selective enrichment of specific miRNAs into exosomes (24, 76). The miRNA repertoire of an exosome is generally different to that of the parent cell, suggesting that exosome packaging is an active process (14). In T cells, for example, Rossi et al. identified a set of 20 miRNAs of which only 2 were differentially expressed in T_H cell-derived exosomes (77). Upon activation primary $CD4^+$ T cells down-regulate their miRNA content. Some of these miRNAs accumulate in exosomes, for example, miR-150, suggesting that the cell may be shedding miRNA as part of a regulation step (70). de Candia et al. quantified the amount of miR-150 present in sera isolated from mice immunized with OVA plus an adjuvant, and reported an increased level of this miRNA in immunized mice as compared to non-immunized mice (70, 78). When they removed $CD4^+$ T cells no elevated miR-150 levels were observed. They next validated this observation using sera collected from adults and children vaccinated with the 2009 pandemic flu (H1N1) vaccine. Similar to the mouse model, they observed that miR-150 was evident in the sera following vaccination, and that this miRNA was associated with lymphocyte derived exosomes. In addition, increased levels of miR-150 correlated with high antibody levels post vaccine, suggesting a link between activation of the adaptive immune responses and expression of a specific miRNAs in exosomes (70, 78). From the adoptive cellular therapy point of view, this data is very exciting as it highlights the possibility of using exosomes to monitor cellular therapies such as Tregs *in vivo*. Given that Tregs produce exosomes only following activation, and in the case of transplantation this will be following recognition of alloantigen presented by donor and recipient DCs, it may be possible to assess Treg viability and function *in vivo* by monitoring Treg exosomes in the blood of transplant recipients. If this is possible Treg exosomes may be unique biomarkers for immune suppression (**Figure 1B**).

As mentioned earlier in addition to miRNA, mRNA, and proteins associated with exosomes can also act as diagnostic tools. For example, in patients with kidney disease CD2AP mRNA was associated with urinary exosomes (79). Several specific proteins have been identified in exosomes isolated from: (1) the urine of healthy individuals (CD24 and Aquaporin 2) (80), (2) sera from cancer patients (MUC1, LRG1, Hsp90a, and RAD21) (81), (3) the placenta (syncytin-1) (82), and 4) from patients with multiple sclerosis (IB4) (83). Taken together, these studies suggest the importance of validating the expression of mRNA and proteins, in addition to miRNAs, in Treg exosomes if unique biomarkers are to be identified.

In conclusion, at present Treg exosomes are still in their infancy with regard to transplantation, either as a therapy or a diagnostic tool. As outlined in this review, several key questions regarding their composition and function need to be addressed. In addition, better isolation and analysis protocols, as well as preclinical models are required before Treg exosomes can make the transition from the lab to the clinic, even for diagnostic purposes. Although some of the ideas presented here are speculative, pursuing the use of Treg exosomes for immune modulation and diagnostic purposes

Table 1 | miRNAs present in exosomes isolated from the sera of patients with specific cancers or following immunization are being used as diagnostic biomarkers.

miRNA identified in exosomes	Cells origin	Reference
miR-150	$CD4^+$ T cells	(70)
miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214	Ovarian cancer	(71)
miR-205, miR-19a, miR-19b, miR-30b, and miR-20a	Lung squamous cell carcinoma	(72)
let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a	Colon cancer	(73)
hsa-miR-31, miR-185, and miR-34b	Melanoma	(44)

within a transplantation setting is timely given that clinical trials are now underway using Treg cells themselves.

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IMMUNOLOGY REVIEW ARTICLE

MicroRNAs affect dendritic cell function and phenotype

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Summary

MicroRNA (miRNA) are small, non-coding RNA molecules that have been linked with immunity through regulating/modulating gene expression. A role for these molecules in T-cell and B-cell development and function has been well established. An increasing body of literature now highlights the importance of specific miRNA in dendritic cell (DC) development as well as their maturation process, antigen presentation capacity and cytokine release. Given the unique role of DC within the immune system, linking the innate and adaptive immune responses, understanding how specific miRNA affect DC function is of importance for understanding disease. In this review we summarize recent developments in miRNA and DC research, highlighting the requirement of miRNA in DC lineage commitment from bone marrow progenitors and for the development of subsets such as plasmacytoid DC and conventional DC. In addition, we discuss how infections and tumours modulate miRNA expression and consequently DC function.

Keywords: dendritic cells; immune modulation; microRNAs

Dendritic cell subsets (human versus mouse)

Dendritic cells (DC) are important antigen-presenting cells for promoting immune responses to pathogens such as bacteria and viruses, as well as for maintaining self-tolerance.[1] These cells link the innate and adaptive immune systems by presenting antigen to T cells, providing co-stimulation and cytokines required for antigen-specific T-cell activation. Many varieties of DC have been described in both human and mouse, each with a particular location, phenotypic morphologies and function.[1–3] To summarize, the major DC categories in the mouse include, conventional/classic DC (cDC), Langerhans DC (LC), plasmacytoid DC (pDC) and the monocyte-derived DC (moDC). The cDC can be subdivided into migratory DC, found in the skin and lymph nodes or tissue-resident DC, found in spleen and lymph nodes. Tissue-resident DC consist of several prominent subsets including (i) the $CD8\alpha^+$ DC, which are important for cross-presenting antigen to $CD8^+$ T cells as well as being the major interleukin-12 p70 (IL-12p70) producer, (ii) the $CD11b^+$ cDC, a heterogeneous population of DC including; $CD4^+$ DC, which are capable of presenting Class II restricted antigens to $CD4^+$ T cells and (iii) pDC, which are the major producer of type 1 interferons during viral infections.[1–3] In man, the equivalent of the mouse $CD8\alpha^+$ DC has been defined, as $CD141^+$ ($BDCA3^+$) DC,[4] as has the pDC subset.[5]

Monocyte-derived DC are derived from monocytes under inflammatory conditions and are closely related to the $CD11b^+$ DC. Murine bone marrow (BM) cells cultured *in vitro* in the presence of granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 develop into what many consider moDC, as do DC derived from human blood monocytes cultured in the presence of these cytokines.[6] Their *in vivo* equivalent has yet to be identified; however, several murine moDC candidates have been described including DC-specific intercellular adhesion molecules-3 grabbing non-integrin (DC-SIGN/CD209a) -positive DC that appear in lymph nodes after Toll-like receptor (TLR) ligand challenge[7] and tumour necrosis factor-α (TNF-α) and inducible nitric oxide synthase-producing DC that appear during pathogen-associated inflammation.[8]

Given the heterogeneity of DC subsets as well as the wide-ranging function of DC, the following question arises, how is DC differentiation and function regulated? Different growth factors, such as cytokines (FLT3 and GM-CSF) and transcription factors (Irf8, E2-2, Id2, E4Bp4, Batf3, Irf4 and Notch-2) control cDC development.[9–11] There is now increasing evidence that microRNA (miRNA) play an important part in 'fine tuning' the

development and function of all DC subsets and this review will focus on recent findings in this exciting field of research.

MicroRNA

MicroRNA are vital for controlling many processes within the immune system including cell differentiation and homeostasis, cytokine responses, interactions with pathogens and tolerance induction.[12] Defects in the action of miRNA are associated with oncogenesis and other diseases.[13–15]

MicroRNA are small, non-coding RNA of around 19–24 nucleotides in length,[16] which function to suppress protein synthesis by binding to complementary 3′-untranslated regions of mRNA[17–19] and either inhibit translation or accelerate mRNA degradation.[19] The process of miRNA synthesis is shown in Fig. 1. Briefly, miRNA are first transcribed into a primary transcript (pri-miRNA) by RNA polymerase II. Pri-miRNA then bind DiGeorge syndrome critical region gene 8 (DGCR8) and undergo processing by the RNAase III enzyme activity of Drosha resulting in hairpin pre-miRNA transcripts.[20–22] The RNase III enzyme, Dicer, further processes these structures in the cytoplasm, following export from the nucleus via Exportin-5. This results in mature, 19- to 24-bp miRNA species, which are incorporated into the RNA-induced silencing complex (RISC) containing the Argonaute (Ago) protein.[23–25] Each miRNA has the ability to inhibit many mRNA, in fact a single miRNA has the ability to affect over 100 genes and one mRNA can be targeted by more than one miRNA.[20,26] MicroRNA can also regulate the expression of other miRNA.[27] For many miRNA the target 3′ untranslated region of specific mRNA have been identified. A summary of some key miRNA involved in DC development and function and their targets are highlighted in Table 1.

A key role for miRNA in regulating T- and B-cell immune cell development, immune homeostasis and controlling adaptive immune responses has been described.[28–31] As for DC, Kuiper *et al.* observed that the conditional depletion of *Dicer* in mouse CD11c^{+} DC did not affect the short-lived resident DC present in lymph nodes or the spleen. However, they observed that both differentiation and function of LC was greatly affected, in as much as the lack of miRNA in these cells resulted in a selective loss of these cells in the epidermis and those that remained lacked the ability to mature and present antigen.[32] Despite this finding, many publications now highlight the importance of miRNA in all murine DC subsets with a role for different miRNA in DC development and function now being well established. In fact, miRNA can 'fine tune' the immune response by inducing apoptosis, affecting homeostasis and changing cytokine profiles of DC.[33,34]

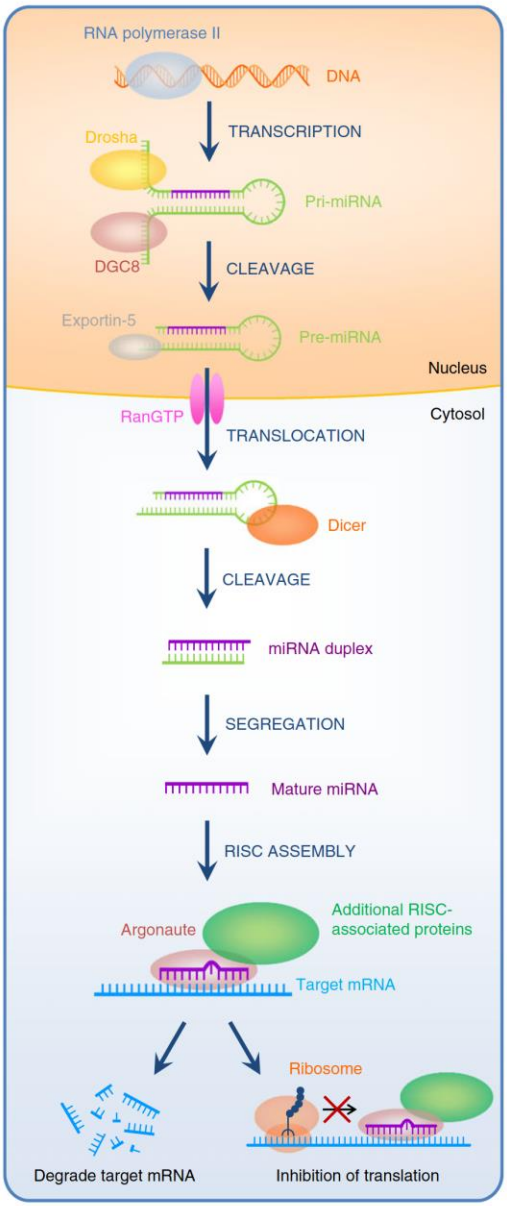

Figure 1. Biogenesis of microRNA (miRNA). In the nucleus, miRNAs are transcribed into primary transcript (pri-miRNAs) by RNA polymerase II. Pri-miRNAs then bind DiGeorge syndrome critical region gene 8 (DGCR8) and undergo processing by the RNAse III activity of Drosha resulting in hairpin pre-miRNA transcripts, which are transported to the cytoplasm via Exportin-5. The RNase III enzyme, Dicer, further processes these structures in the cytoplasm resulting in mature, 19- to 24-base-pair miRNA species, which are incorporated into the RNA-induced silencing complex (RISC) containing the Argonaute (Ago) protein. miRNA recognize the 3′ untranslated region of mRNA as part of the RNA-induced silencing complex (RISC).

Table 1. MicroRNA (miRNA) regulate production of cytokines, differentiation and homeostasis of many dendritic cell subsets via affecting specific targets

miRNA	Predicted/identified targets	References
miR-155	TAB 2, PU.1, SOCS1, SHIP1, KPC1, *csf1* gene, c-FOS, DC-SIGN, C/EBPβ	33,37,44,45, 47–49,71,82
let-7	BLIMP SOCS1	50,70
miR-29a	P42.3 Lipoprotein lipase	83,84
miR-142-3p	IL-6	51
miR-125a and miR- 99a	KLF13	36,85
miR-148a and 148b miR152	Calcium/calmodulin-dependent protein kinase IIa	54
miR-29b and miR-29c	Bcl-2 and Mcl-1	58
miR-146a	IRAK1, IRAK2, TRAF6, TLR-4	41,86
miR-126	*Tsc1* (which encodes a negative regulator of the kinase mTOR)	52
miR-34a	JAG1, WNT	38
miR-142		1,10
miR-21	Jag1, PDCD4, IL-12p35	33
miR-221	$P27^{kip1}$	33,39
miR-22	Irf8	40
miR-23b	Notch 1 and NFKB	62
miR-107	IL-23p19	66
miR-301a	PTEN	69,86
miR-451	YWHAZ/14-3-3z protein levels	64
miR-30b	Notch1	36

MicroRNA and DC differentiation

Dendritic cells are derived from haematopoietic stem cells (HSC) present in BM. HSC differentiate into myeloid progenitor cells and myeloid DC progenitor cells. The latter are the precursors of cDC progenitors, which upon leaving the BM seed peripheral secondary lymphoid tissues and non-haematopoietic tissues, where they give rise to the aforementioned immature DC subsets, which upon TLR stimulus, mature. Differential expression of miRNA has been observed throughout the developmental process of murine DC from HSC to immature and mature DC.[35] Su *et al.*[36] compared the miRNA profile of BM-derived DC (BMDC) HSC, immature and mature DC using next-generation deep sequencing. These authors demonstrated that 391 miRNA were differentially expressed during DC differentiation.[36] However an overlap in miRNA expression between each developmental stage was also observed, for example miR-132 and miR-147 were highly expressed in immature and mature DC but not found in HSC.[36] miRNA profiles changed significantly, between HSC and immature DC. GM-CSF expanded, immature and lipopolysaccharide-activated murine moDC also display distinct miRNA expression profiles.[37] The requirement for miRNA for human moDC development from progenitors has also been shown with miR-21 and miR-34a both being implicated in this process.[38]

The observation that miRNA profiles vary greatly between progenitor and DC also extends to the different DC subsets.[39] Mildner *et al.*[10] observed that whereas some miRNA were highly expressed in all subsets of DC, for example miR-125, let-7 and miR-21, murine pDC and splenic cDC (both $CD8\alpha^+$ and $CD4^+$) have defined clusters of miRNA signatures. Moreover, miR-22 was found to be highly expressed in cDC ($CD4^+$, $CD8\alpha^+$ and $CD4^-$ $CD8^-$) compared with pDC. Over-expression of this miRNA in DC progenitors both *in vitro* and *in vivo* preferentially expanded the $CD11c^+$ $CD11b^+$ $B220^-$ cDC subset.[40]

Development and maintenance of $CD4^+$ DC has been linked to expression of miR-142.[10] The miR-142 is highly expressed in FLT3-dependent $CD4^+$ DC, but not $CD8\alpha^+$ or $CD4^-$ $CD8\alpha^-$ DC. Mice deficient in miR-142 have a 60% reduction in Class II $CD11c^{hi}$ DC owing to an increase in $CD4^+$ DC apoptosis. In addition, miR-142-deficient BM cells failed to develop into $CD4^+$ DC *in vitro*, in the presence of FLT3L; however, there was no inhibition of $CD8\alpha^+$ DC development. Interestingly, the loss of miR-142 in these mice only affected splenic $CD4^+$ DC development, the gut $CD4^+$ DC equivalents, the $CD103^+$ $CD11b^+$ DC, were found at normal numbers.[10]

The development of pDC is also regulated by miRNA. Inhibiting miR-221 in BMDC progenitors led to the differentiation of pDC rather than cDC.[33,39] Development of pDC has also been shown to require miR-126, which is highly expressed in both mouse and human pDC. Increased apoptosis of these cells was observed in the absence of this miRNA, suggesting that it is important for pDC survival.[32,33] Another miRNA, miR-146a, has also been shown to affect pDC survival; over-expression of this miRNA in a pDC cell line induced apoptosis in these cells. This may reflect the fact that miR-146a blocks TLR-induced nuclear factor-κB (NFKB) activity by targeting IL-1 receptor-associated kinase 1 (IRAK1), leading to down-regulation of anti-apoptotic genes.[41]

MicroRNA and DC function

Cell intrinsic factors that form part of the anti-viral defence system, such as pattern recognition receptors (e.g. TLR, nucleotide-binding oligomerisation domain (NOD)-like receptor (NLR)), as well as cytokines, lipids, viruses, bacteria, parasites and tumours can modify the miRNA expression within DC.[34,35,42] Each of these different stimuli was shown to induce or decrease expression of miRNA that influence, either positively or negatively, the ability of DC to process antigen, mature (expression of

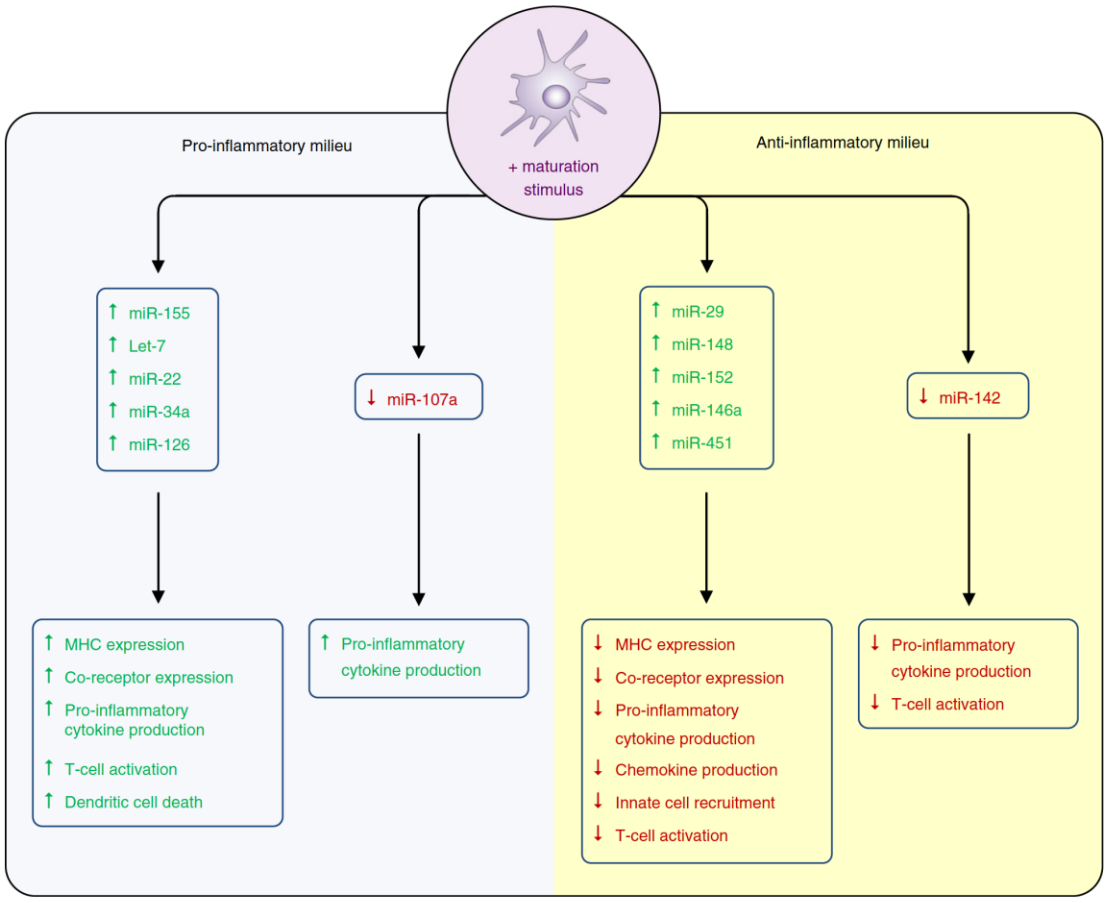

Figure 2. MicroRNA (miRNA) expression during dendritic cell (DC) maturation. Activation of DC results in either an up-regulation or down-regulation of specific miRNAs that can modulate pro- or anti-inflammatory responses as well as T-cell activation and DC survival.

MHC, CD40, CD80, CD86 and DC-SIGN expression) and function (cytokine production and T-cell activation). Some of these miRNA are highlighted next and their effects on DC are summarized in Fig. 2.

MicroRNA associated with DC maturation and function (miR-155, Let-7i and miR-126)

miR-155

Expression of miR-155 is induced rapidly in both human and mouse DC following maturation induced by either TLR activation (for example by dsRNA), cytokines [for example IL-1β, TNF-α and interferon-γ (IFN-γ)] as well as lipids (such as oxidized low-density lipoproteins and low-density lipoproteins).[43] Expression of miR-155 by murine DC has been shown to be important for both their maturation and function, as highlighted by the observation that miR-155-deficient mice display impaired immune responses to pathogens.[44,45] This observation being due in part to reduced expression of CD40 and CD86 following TLR activation.[43] The DCs isolated from miR-155-deficient mice also have an impaired ability to activate antigen-specific T cells.[46] Recently, the mechanism by which miR-155 modulates the ability of DC to activate T cells has been described.[43] Dunand-Sauthier *et al.* found that the arginase, Arg-2, is a direct target of miR-155. Whereas miR-155-deficient BMDC have abnormally high levels of Arg-2 an increased expression of miR-155 in murine BMDC was associated with down-regulation of its expression. As arginine depletion by Arg-2 facilitates impaired T-cell proliferation, miR-155-induced repression of Arg2 expression appears critical for DC to activate T cells by controlling arginine availability in the extracellular environment.[43]

Increased levels of miR-155 in human moDC has been linked to DC co-receptor expression levels. The transcription factor PU.1, which regulates the expression of molecules such as DC-SIGN, is a direct target of miR-155.[47] During DC maturation the increased expression of miR-155 results in decreased PU.1 levels and a subsequent reduction in DC-SIGN mRNA.[47] The miR-155 has also been shown to modulate cytokine release.[48,49] In human moDC, Ceppi *et al.*[48] observed that inhibiting miR-155 expression in lipopolysaccharide-activated DC resulted in an increase in pro-inflammatory cytokine gene expression with IL-1α, IL-1β, IL-6, TNF-α and IL-23 being among the up-regulated genes found. These authors suggested that this miRNA could be an inhibitor of the inflammatory response given that down-regulation of these pro-inflammatory molecules occurred when miR-155 expression levels increased.[48]

Let-7i

miR-155 is not the only miRNA associated with DC maturation. For example, up-regulation of the miRNA let-7i in DC following TLR4 activation is important for normal DC maturation.[50] When inhibited, expression of the co-stimulatory molecules CD80 and CD86, as well as pro-inflammatory cytokine production, were decreased. In addition, T-cell responses to antigen presented by DC were also reduced.[50] The increased levels of miRNA let-7c and miR-155 in DC following maturation resulted in a pro-inflammatory phenotype, through the inhibition of the suppressor of cytokine signalling 1 (SOCS1), an inhibitor of Janus kinase/signal transducer and activator of transcription signalling after TLR activation.[51]

miR-126

Plasmocytoid DC maturation and function is also regulated by miRNA.[52] Agudo *et al.*[52] observed that miR-126 was highly expressed in both murine and human pDC compared with the cDC subsets. Mice lacking miR-126 were found to have reduced pDC-mediated activation, migration and IFN-γ release following TLR activation with CPG-A, suggesting that this miRNA was important for pDC function.[52] These authors suggest that miR-126 regulated pDC function by directly targeting the mTOR pathway, which is required for TLR signalling by pDC,[53] by controlling mRNA expression of tsc-1 a negative regulator of this pathway.[52]

MicroRNA that prevent DC maturation (miR-148, miR-142, miR-146a and miR-29a)

Although some miRNA are increased in DC following activation, their expression has a negative effect on DC maturation. For example, miR-148 is up-regulated in murine BMDC following TLR4 activation. Increased levels of this miRNA led to reduced MHC Class II expression, inhibition of pro-inflammatory cytokine secretion and decreased DC-mediated $CD4^+$ T-cell expansion.[54] In addition, in human DC, miR-29 was found to be up-regulated in response to NOD2, a cytoplasmic pattern recognition receptor signal, which led to the down-regulation of the pro-inflammatory cytokine IL-23 by targeting IL-12p40 and IL-23p19.[55]

As mentioned earlier, pDC effector function (e.g. cytokine production) and the expression levels of co-stimulation molecules are also controlled by miRNA. Expression of miR-146a is induced by TLR ligation (TLR7/9) in human pDC.[41] Increased expression of this miRNA (via lentiviral transduction) in a human pDC line impaired TLR-mediated maturation by inhibiting key components of the nuclear factor-κB pathway. In addition, increased miR-146a expression inhibited the up-regulation of CD40, CD80, CD86, HLA-DR and CCR7 molecules and the production of pro-inflammatory cytokines (IL-6 and IFN-γ) such that pDC-induced allogeneic T-cell responses were inhibited.[41]

miR-142

Maturation of DC also results in down-regulation of miRNA. miR-142 is constitutively expressed in immature BMDC and following lipopolysaccharide activation its expression is decreased.[10,51] Down-regulation of miR-142 was found not to affect activation markers such as CD40, CD80 and CD86, all of which increase following TLR activation in its absence. However, despite these maturation changes, DC isolated from miR-142-deficient mice failed to induce a $CD4^+$ T-cell response compared with normal DC. This observation was attributed to miR-142 directly controlling IL-6 mRNA and IL-6 production following lipopolysaccharide activation.[51]

MicroRNA that affect antigen presentation (miR-150 and miR-223)

In addition to regulating cytokine production and co-receptor levels, miRNA can affect the ability of DC to present antigen. Epidermal LC from miR-150-deficient mice have reduced soluble antigen cross-presentation abilities[56] while deletion of miR-223 increases the capacity of LC to cross-present.[57] How both of these miRNA affect the antigen presentation pathway has yet to be elucidated; however, miR-150-deficient LC do not have impaired phagocytic capacities, suggesting that this miRNA may affect antigen processing. Whether miRNA affect antigen presentation in different DC types has yet to be studied.

MicroRNA that affect DC survival (miR-155, miR-146, miR-126, miR-29b and miR-29c)

MicroRNA expression has also been linked with DC survival. BMDC derived from miR-155-deficient mice survived longer than DC isolated from normal BM after lipopolysaccharide activation, whereas over-expression of miR-155 in DC led to increased apoptosis of these cells.[33] KPC1, which ubiquitinates $p27^{kip1}$ for degradation, is a target of miR-155. The miR-155 inhibits KPC1 expression leading to enhanced $p27^{kip1}$ levels and ultimately in DC death.[33] Thereby expression of this miRNA following stimulation may help to resolve an immune response by inducing the death of antigen-presenting cells. Plasmacytoid DC apoptosis and cell survival is also regulated by miRNA, including miR-146,[41] miR-29b and miR-29c,[58] as well as miR-126.[52]

MicroRNA and DC in tolerance induction

Immature DC, cytokine (transforming growth factor-β; TGF-β) and/or drug-treated 'tolerogenic' DC can induce T-cell tolerance. 'Tolerogenic' DC produce less pro-inflammatory cytokines in favour of cytokines such as IL-10 and TGF-β.[59] Several miRNA have been shown to inhibit pro-inflammatory cytokine production, including miR-21 (inhibits IL-12p35 production),[60] miR-10 (inhibits IL-12/IL-23p40 chain)[61] and miR-148a/152 which suppress both IL-6 and IL-12 production via calcium/calmodulin-dependent protein kinase IIα (CaMKIIα), an effector of calcium signalling pathways.[54]

Several miRNA have been linked with a 'tolerogenic' DC phenotype (Fig. 3). Su *et al.*[36] observed that miR-30b is significantly up-regulated in 'tolerogenic' DC (mature DC plus TGF-β) both *in vitro* and *in vivo*. Over-expressing miR-30b in DC led to increased IL-10 and NO production while inhibiting it reduced both. In addition, miR-125a and miR-99a expression increased in 'tolerogenic' DC in this study.[36] miR-23b has also been associated with a 'tolerogenic' DC phenotype. Expressing miR-23b in mouse BMDC and human moDC, via transfection, resulted in DC with reduced IL-12 but increased IL-10 production capacity, as well as reduced Class II, CD80 and CD86 expression. Increased expression of Foxp3 was seen when $CD4^+$ T cells were co-cultured in the presence of miR-23b expressing DC.[62] Both Notch1 and nuclear factor-κB are inhibited by miR-23b.[62]

Stumpfova *et al.*[63] analysed the expression of miRNA in 'tolerogenic' human moDC (IL-10- and TGF-β-treated) versus lipopolysaccharide-treated, IFN-γ-treated DC and immature DC. These authors showed that 27 miRNA, including miR-17, miR-133b and miR-203, were specifically increased in 'tolerogenic' DC when compared with mature DC. They also found four miRNA that were down-regulated in 'tolerogenic' DC, miR-99b, miR-135a, miR-147 and miR-214.[63] Low levels of the miRNA let-7 in human moDC have been shown to favour the expansion of regulatory T cells, following interaction with these DC, again linking miRNA and tolerance induction.[50]

MicroRNA and DC during infections/tumours

Profiling murine splenic DC infected with influenza A virus *in vitro* revealed that miR-451 was induced by this ssRNA virus and not by dsRNA or lipopolysaccharide. Interestingly, following infection with this virus, myeloid cells present in the lung had increased expression of this miRNA. Expression of miR-451 in splenic DC reduced the production of a specific set of cytokines and chemokines including IL-6, TNF-α, IFN-γ, macrophage inflammatory protein-1α, CCL5 (involved in recruitment of T cells, eosinophils and basophils), CCL3 (involved in

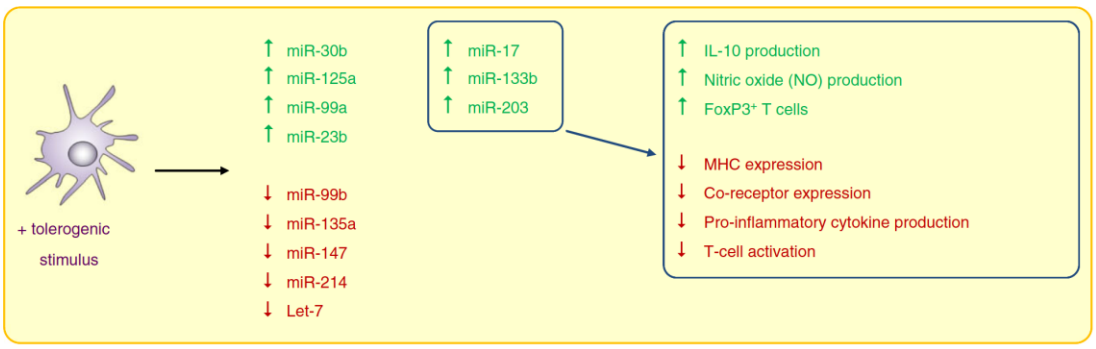

Figure 3. MicroRNA (miRNA) species linked with 'tolerogenic' dendritic cells (DC). Immature DC, cytokine (transforming growth factor-β) and/or drug-treated 'tolerogenic' DC can induce T-cell tolerance. 'Tolerogenic' DC produce less pro-inflammatory cytokines, in favour of cytokines such as interleukin-10, they also produce NO and can induce $FoxP3^+$ T cells. Modification of specific miRNA has been linked to this 'tolerogenic' DC phenotype and function.

monocyte and neutrophil recruitment). This miRNA did not affect IL-10 or IL-1β production. Interestingly, inhibiting this miRNA had no effect on the expression levels of Class II and CD80 levels following activation. Taken together, it appears that viral infections may modulate miRNA expression to create an anti-inflammatory environment.[64]

Likewise, helminth worm antigens have also been shown to create an anti-inflammatory environment by modulating miRNA in human DC. Exposure of human moDC to secreted antigen from *Taenia crassiceps* reduced pro-inflammatory cytokine and chemokine production by inhibiting lipopolysaccharide-induced miRNA let-7 expression.[65]

Bacteria, including those of the intestinal microbiota, have been shown to have both a positive and a negative effect on host miRNA expression.[61,66–68] Exposing germ-free mice to gut microbiota led to down-regulation of miR-10a in DC, and an increase in the pro-inflammatory IL-23p40 subunit.[61] In addition, BMDC exposed to gut microbacteria such as *Escherichia coli* and flagellated A4 commensal bacteria have significantly lower miR-107a levels compared with untreated DC.[66] Microbiota were also found to modulate miR-107a expression in gut CD11c$^+$ myeloid cells leading to greater IL-23p19 production by these cells.[66]

Recently, it was reported that tumours modify miRNA expression in DC, creating an immune-suppressing and tumour-promoting environment.[69] MicroRNA linked with tumour immune modulation such as miR-21, miR-222, miR-28 and miR-301a were up-regulated in DC exposed to tumour antigens. Over-expression of miR-301a in FLT3L-expanded BMDC did not affect Class II or CD80, CD86 or CD40 expression but inhibited IL-12, IL-6 and TNF-α production by DC and modulated T-cell responses. Although the interaction with miR-301a expressing DC did not affect antigen-specific CD4$^+$ and CD8$^+$ T-cell proliferation, cytokine release was modified; decreased IFN-γ from CD8$^+$ CTL and CD4$^+$ T cells while increased IL-3 and IL-17 were observed.[69]

What controls microRNA expression?

Several factors can regulate the expression of miRNA in DC, including transcription factors. For example, BLIMP affects the level of let-7c miRNA expression and miR-142, which has its own promoter, is targeted by the transcription factor PU.1.[51,70]

MicroRNA can also control the expression of other miRNA. For example, miR-155 has been shown to regulate the expression of miR-142. Increasing miR-155 expression following TLR ligation leds to reduced miR-142 expression, due to miR-155 targeting the PU.1 promotor. PU.1 is required for miR-142 expression.[51] Conversely, knocking down miR-155 resulted in an increased miR-142 expression.[51,62] Recently, it has been suggested that miR-155 is a 'master' miRNA regulator in DC.[71] Comparing the miRNA profile in miR-155-deficient with miR-155 expressing DC, following a maturation stimulus, revealed miR155 'dependent' and 'independent' miRNA. For example, miR-445-3p is induced following DC maturation only in the absence of miR-155 whereas miR-210-3p is not induced unless miR-155 is present. In addition, immature DC from miR-155-deficient mice lack miR-210-3p. It has been hypothesized that miR-155 regulates transcription factors, such as CCAAT/enhancer binding protein-β for example, that then bind to other miRNA promoters, such as miR-455, leading to up/down-regulation of these molecules.[71]

Intercellular transfer of microRNA

MicroRNA changes in DC may not always be due to intrinsic factors.[72–75] In fact the intercellular transfer of miRNA between cells has been described through two pathways, gap junctions (GJ) and exosomes. At present the transfer of miRNA to DC via GJ has not been elucidated; however, this phenomenon has been described for several other cell types. Lim *et al.*[73] demonstrated that miRNA could be transferred via GJ from BM-derived stromal cells into breast cancer cells where they caused a reduction in CXCL12 expression. In addition, Katakowski *et al.*[72] elegantly showed miRNA transfer via GJ between miR-67-expressing gliosarcoma cells and target cells expressing a luciferase reporter containing an miR-67 binding site. They observed that following co-culture, luciferase expression was reduced, an effect that was reversed by the presence of carbenoxolone, a GJ uncoupler.[72,76] Recently Aucher *et al.*[77] found that miRNA were transferred from human macrophages to a hepatocarcinoma cell line. These studies suggest first that, miRNA transfer between cells can occur via GJ and second, that transferred miRNA are functional. Given that GJ formation occurs between T cells and DC during interaction at the immune synapse[78] the possibility that the intercellular transfer of miRNA occurs between these cells warrants further research.

MicroRNA are also found in exosomes released by cells such as DC and T cells.[74,79] The miRNA profiling of DC exosomes found that miRNA expression differs with the maturation status of the DC.[75] Interestingly, miRNA present in DC-derived exosomes can be transferred to other DC *in vitro* and *in vivo* where they are functional.[75] Recently, Mittelbrunn *et al.* observed that T-cell-derived exosomes also contain specific miRNA, which can be transferred to antigen-presenting cells, leading to modification of cell function.[74,80,81] T-cell-derived exosomes are released during immune synapse formation, suggesting that during immune interactions directed release of miRNA-laden vesicles to DC during immune recognition may lead to immune modulation.[80]

Concluding remarks

It is clear that miRNA expression in BM progenitors drives DC differentiation, and in immature/mature DC miRNA expression helps to shape the adaptive immune response as well as resolve it through inducing death of the DC. Although miRNA plays an important role in DC function following interaction with infectious agents such as viruses, bacteria and parasites, little is known about whether cells of the immune system modulate DC miRNA during infection and tolerance induction either directly or via exosome release. Given the unique role of DC within the immune system, presenting antigen and shaping immune responses, understanding how cells of the adaptive immune system regulate DC miRNA is of vital importance.

The observation that both pathogens and tumours have evolved strategies that can modulate DC miRNA, creating either a non-inflammatory or inflammatory environment, is of clinical relevance and warrants further investigation in defined disease states.

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Disclosures

The authors declare no financial or commercial conflict of interest.

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